ANIMAL REPRODUCTION

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Contents

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Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Gramado, RS, Brazil, August 20th to 23rd, 2015, and 31st Meeting of the European Embryo Transfer Association (AETE); Ghent, Belgium, September 11th and 12th, 2015

From the SBTE President

Dear Colleagues,

We are pleased to announce the Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society (SBTE). We hope you enjoy the meeting and take advantages of the opportunity to gain new scientific insights, renew friendships and make new contacts.

A novelty for this year, due to the effort of our scientific director Dr Roberto Sartori, is that the annual proceedings will be published with the European Society of Embryo Transfer (AETE) in Animal Reproduction. This is a great achievement since the will allow the proceedings to be available to a wider audience. In addition, it will strengthen collaboration between AETE and SBTE by sharing knowledge and experience more effectively. We are grateful to the Animal Reproduction editor and staff for the support received.

Similar to the previous year, the event was planned considering both the interests and needs of field technicians, academics and scientists. The Chairman of the SBTE Scientific Committee along with the members of the Program Committee has brought together diverse topics and speakers to stimulate thoughts and discussion. In addition to the traditional plenary, we will have thematic modules divided into "SBTE Science" and "SBTE Technology". Roundtables to discuss relevant issues are also part of the program, along with the students and professionals awards.

We received over 260 abstract submissions, which will be presented in posters sessions accompanied by a *Serra Gaucha* wine.

As in the past, this meeting depends on the full support and involvement of our partner companies as well as our sponsors - CNPq and CAPES, to whom we are deeply grateful. I also want to thank all the speakers who have agreed to attend this meeting and share their last findings with us. My special thanks for all SBTE Board of Directors and collaborators, whom have turned this meeting in to a reality.

We look forward to seeing you in Gramado.

Margot Alves Nunes Dode, PhD
President
(2014-2015)

Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Gramado, RS, Brazil, August 20th to 23rd, 2015, and 31st Meeting of the European Embryo Transfer Association (AETE); Ghent, Belgium, September 11th and 12th, 2015

From the SBTE scientific committee chair

Greetings and welcome to the 29th SBTE Annual Meeting! The Scientific Committee is very happy with this new challenge, which is the joint publication with the proceedings of the 31st AETE Annual Meeting. Therefore, we thank Jo Leroy, Urban Besenfelder, Frank Becker and other members of the AETE Executive Board, as well as the scientists who collaborated with revisions of the AETE abstracts and manuscripts. For the 2015 SBTE, we maintained most of the program structure as we did in 2014. We invited world-class speakers to lecture about up-to-date information on embryo technology-related fields. The novelty for this year are the round tables in hot topics, in which several speakers will have a short presentantion and more time for debate. We will encourage the audience to participate and engage more on debates. The opening ceremony will have the traditional talk from the IETS President, plus an exciting lecture about gamete derivation from embryonic stem cells. The main program will have three plenary sessions, and six sessions: Spermatozoa, Oocyte, and Embryo ("SBTE Science"), and Embryo Production, Embryo Development and Cryopreservation, and Health ("SBTE Technology"). For the plenary sessions, speakers were carefully picked to deliver contents of general interest to the society. In the other sessions, attendees will choose between two concurrent sessions, one focused on more basic aspects ("SBTE science") and the other emphasizing the more applied aspects ("SBTE technology") of that session's topic. Special emphasis must be directed to the pre-conference workshops. We will have five workshops, with novelties. 1. Artificial Insemination (focusing on beef cattle, this year); 2. GIFT: Genomic selection for reproductive traits (showing an overview of GIFT Consortium – Brazil and Denmark); 3. The III Symposium of the South American Research Consortium on Cloning and Transgenesis in Ruminants; 4. The In Vitro Fertilization Workshop, that will discuss several topics, including health issues, sexed semen, and the international market and embryo import/export; and 5. The Workshop on Andrology and Semen Technology. The SBTE wants to thank all members that sent their best work to be presented at this meeting. For the poster session, we will have all presentations at the same time, but for a longer period of time, for the attendees to be able to discuss in detail all interested data. Finally, SBTE wants to acknowledge the speakers, for putting a lot of effort on the preparation of excellent manuscripts and lectures to be delivered at this year's meeting. We also want to thank all the SBTE team and especially, the Scientific Committee and all the reviewers that worked tirelessly and enthusiastically 24/7. This list includes all folks from the SBTE administrative board, abstract session coordinators, abstract reviewers, manuscript reviewers and scientific editors. Once again, SBTE thanks the editor and staff at the Animal Reproduction journal and the Colégio Brasileiro de Reprodução Animal, for their collaborative spirit and instrumental help on putting together this year's meeting proceedings. We hope you find this volume informative and useful. See you in Gramado!

Roberto Sartori, PhD
Chairman of the SBTE Scientific Committee
(2014-2015)

Proceedings of the 29th Annual Meeting of the Brazilian Embryo Technology Society (SBTE); Gramado, RS, Brazil, August 20th to 23rd, 2015, and 31st Meeting of the European Embryo Transfer Association (AETE); Ghent, Belgium, September 11th and 12th, 2015

Letter from the President of the AETE to introduce the 31st Scientific Meeting in Ghent, Belgium 2015

Dear Colleagues, dear friends

This is to invite you to participate in the 31st Annual Scientific Meeting of the AETE (ASSOCIATION EUROPEENNE DE TRANSFERT EMBRYONNAIRE), which will be held in Ghent, Belgium on the 11th and 12th of September 2015.

The main objectives of the AETE are to facilitate the diffusion of knowledge and the implementation of new methods in the field of embryo transfer and associated techniques. This is achieved through scientific, technical and practical European forum exchanges and the organisation of an annual meeting which gathers both academic and scientific representatives as well as technicians, veterinary practitioners and students.

The AETE aims to create a European scientific and practical pole dedicated to international exchange concerning mammalian embryos by creating working groups which will generate information/issues susceptible to be used by the European commission in the fields of agriculture and/or by veterinary medicine.

The AETE gathers the best European experts in the fields of animal reproduction and embryo biotechnologies. And this year - the European ET-family is assembled here in Ghent. However, for the first time in our history we are connected virtually and we are unified in spirit with our Brazilian colleagues from the SBTE. We will now collaborate with the Brazilian Society of Embryo Transfer to have our proceedings published in a joined special issue of Animal Reproduction. Many thanks to Jo Leroy, Roberto Sartori and to the Editorial Board of Animal Reproduction.

The SBTE meeting takes place at the end of August while the meeting of the AETE is scheduled at the second week of September. This is very close however the distance is more than 10.000 km between both venues. The joint publication will help to create strong bridges between both organizations. It is an extra boost for the journal as now much more people in Europe will also read it. The AETE has now the possibility to get access to a respected journal. The collaboration between SBTE and AETE will be furthermore strengthened, sharing knowledge as well as scientific and practical methods. It is furthermore great to see that both SBTE and AETE give the practitioners a center stage place in their society. I am convinced that we will open a new chapter of collaboration and we are already very enthusiastic about it.

The 2015 program of the AETE is very promising and inspiring. We will have four invited lectures framed by 15 short oral communications, the long-established student competition and two workshops. One workshop will be leaded by Katrin Hinrichs from the States to continue the suspense moment from the new established preconference to our regular meeting. Besides we will have an interesting and very practical related workshop chaired by Martin Gehring from Germany about the evaluation of bovine embryos. For the first time we incorporated a new Preconference Workshop into our program. The focus is on equine reproduction and is titled “A week of life of an equine embryo” and is moderated by Ann van Soom from Belgium. The other exciting news is that we installed the STUDENT´s Breakfast within our AETE Meeting. We want to strengthen the togetherness of the students and our organization. A special honor and pleasure for me will be to overhand our 2015 AETE medal to the “grandfather” of the AETE - Michel Thibier. We are curious to follow his lecture.

In general we believe we have an outstanding program for 2015 designed to meet everyone´s interest and needs as well as connected in spirit with our Brazilian colleagues.

Kind regards

Frank Becker
President AETE

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Multiparametric flow cytometry: a relevant tool for sperm function evaluation

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Abstract

Nowadays in human and animal andrology flow cytometry is recognized as a robust tool for the evaluation of sperm quality and function. However, in this particular field, this technique has not reached the sophistication of other areas of biology and medicine. In recent years more sophisticated flow cytometers are being introduced in andrology laboratories, and the number of tests that can be potentially used in the evaluation of the sperm physiology has increased accordingly. In this review recent advances in the evaluation of sperm will be discussed; representing new techniques in flow cytometry, many of them able to measure simultaneously, in a single test, different degrees of damage in different sperm regions and/or changes in functionality.

Keywords: multiparametric flow cytometry, sperm flow cytometry.

Introduction

The ultimate goal of semen analysis is to determine the fertility of a sire; depending of individual value of each sire, semen analysis may determine whether a sire is simply eliminated as a semen donor, or will receive treatment to improve his fertility. While the definitive proof of fertility is the offspring born from the sire, robust laboratory test are needed. Fertility trials are expensive and time consuming, moreover while in particular species such as pigs and bulls can be relatively easy to perform, in other species this may result much more complicated. Understanding sperm function is an absolute pre-requisite for fertility evaluation. Spermatozoa are terminal fully differentiated cells, with functions that ought to be expressed at specific time intervals, and also have to be able to support dramatic changes in their environment in their journey from the tail of the epididymis to the oviduct. Moreover must have the capacity to readily respond to specific signals originated in the female genitalia. In a landmark paper by Amann and Hammerstedt (1993) the complexity of sperm evaluation was clearly underlined, and addressed the need to evaluate the multiple functions and attributes that the spermatozoa must fulfill to reach and fertilize an oocyte. More recently the discovery of the heterogeneous nature of the ejaculate may introduce the need to develop methods to identify the fertilizing population within a given sample. Classical sperm evaluation implies in most circumstances the evaluation of single attributes, i.e. morphology, sperm number, subjective motility. More recent developments, like CASA evaluates attributes such as sperm velocities that shall depend of the correct function of different sperm attributes, i.e. adequate source of energy and fully functional metabolic pathways. However evaluation of multiple attributes with a single or few tests is not common practice yet.

Flow cytometry in sperm evaluation

One important aspect of sperm evaluation is to born in mind the representativeness of the sample analyzed with respect of the whole ejaculate. In this respect, has been determined that several thousands of spermatozoa is preferred for the sperm analysis, unfortunately conventional semen analysis usually evaluates a few hundred sperm, and even more elaborated analysis, involving fluorescence microscopy and computer assisted sperm analysis, share the same limitation. Flow cytometry was introduced in semen analysis back in the late 70s early 80s. When introducing the terms “flow cytometry and sperm” in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) 1839 papers result, with the first two ones published in 1977 (van Dilla *et al*., 1977) and 1978 (Meistrich *et al*., 1978). Flow cytometry allows a better representation of the sample analyzed, at least numerically, since with this technique is easy to evaluate many thousands of spermatozoa in a few seconds. Since the early use of flow cytometry for DNA analysis, fluorescent methods were introduced in the 90s to monitor membrane integrity, and more recently mitochondrial membrane potential, oxidative status, membrane fluidity and permeability, lipid peroxidation, and tyrosine phosphorylation of sperm proteins, among other assays, have been developed (Peña *et al*., 2005b; Peña, 2007; Ortega Ferrusola *et al*., 2009a, b). Recent reviews (Martinez Pastor *et al*., 2010; Petrunkina and Harrison, 2011, 2013) have addressed flow cytometry techniques currently in use in veterinary andrology, and the reader is referred to them; here we will focus in new possibilities that flow cytometry offer for the simultaneous evaluation of different sperm compartments and functions.

Multiparametric flow cytometry

Multicolor analytical approaches are widely used in different fields of medicine and biology. If these procedures could be adapted to sperm assessment, multiple attributes on individual sperm cells could be rapidly evaluated. This will also introduce a solution to one the major drawbacks that historically has had sperm

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evaluation, the problem of not being able to test multiple attributes simultaneously at the single cell level. Two to three lasers systems have become more affordable, and now are entering in the andrology laboratories. It has been proposed that this systems would allow simultaneous assessment of all major parameters covering the route to fertilization provided that careful selection of probes, correct optical configuration and accurate multicolor compensation are introduced (Petrunkina and Harrison, 2011). Equipment having three excitation lasers has been recently incorporated to the laboratory of the author (MACSQuant Analyzer 10, Miltenyi, Biotech, Bergisch Gladbach, Germany). This is a compact flow cytometer equipped with violet (Excitation wavelength 405 nm), blue (Excitation wavelength 488 nm) and red (Excitation wavelength 635 nm) lasers, and 8 photomultiplier tubes plus FSC and SCC detectors. The excitation and emission wave lengths that this system provides, allows the use of combinations of probes that can assess simultaneously multiple parameters in a large number of spermatozoa (usually >40,000). Two panels (and combinations) are now routinely used in our laboratory to measure simultaneously membrane integrity, functional changes in membrane permeability and mitochondrial membrane potential or oxidative stress, and at the same time gate out debris easily. Other panels are designed to determine simultaneously membrane integrity, caspase activity and mitochondrial membrane potential or oxidative stress. The first panel involves the use of Hoechst 33342, propidium iodide or ethidium homodimer, YoPro-1 and Mitotracker deep Red or CellRox Deep Red reagent. Hoechst 33342 can be excited with the violet laser, YoPro-1 and ethidium homodimer are excited with the blue laser and Mitotracker deep red is excited with the red laser. Correct compensation for spectral overlap is critical in multicolor protocols; unstained and single stained controls are mandatory. The first advantage of this combination of probes is that "alien" particles can be easily gated out from the analysis. Only Hoechst 33342 positive events are considered spermatozoa, avoiding errors inherent to gating decisions based in FSS and SCC characteristics. Multiple dot plots can be created, a dot plot combining Hoechst 33342 with propidium iodide or Ethidium homodimer can be used to determine the percentage of live and dead spermatozoa in the sample, being an alternative to the traditional use of SYBR-14/PI. Combinations of YoPro-1/propidium iodide and Hoechst 33342/YoPro-1 can be used to detect changes in membrane permeability; these variations may be related either to early sperm damage or being capacitation related changes. Finally mitochondrial membrane potential is assessed with Mitotracker deep Red, or oxidative stress assessed with Cell Rox Deep red reagent. Hierarchical gating can be applied to determine, for example, sources of reactive oxygen species (ROS) in specific sperm populations. An example of one of these panels is given in Fig. 1.

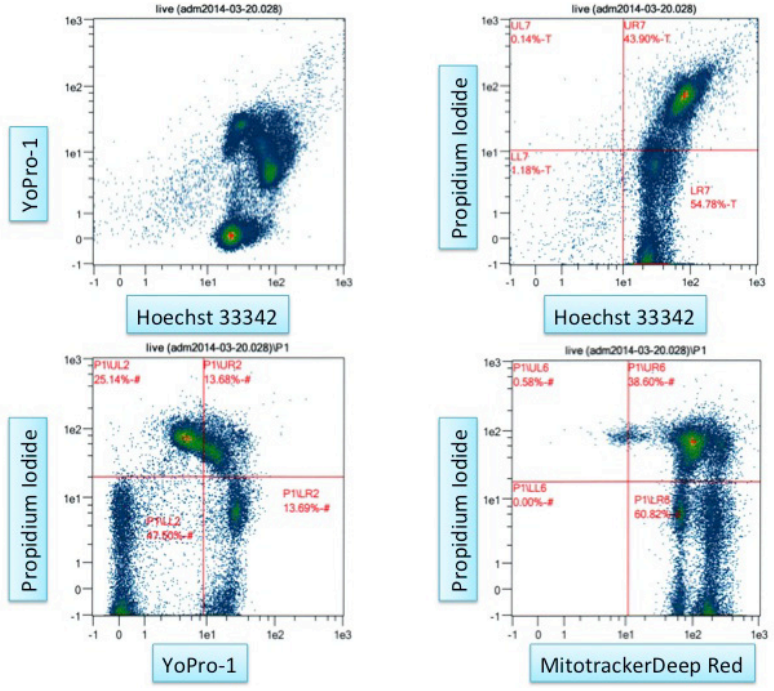

Figure 1. Example of a multicolor panel for simultaneous evaluation of different sperm parameters. Spermatozoa are stained with Hoechst 33342, YoPro-1, Propidium Iodide and Mitotracker deep Red. In the upper right dot plot information of the percentage of live spermatozoa are given thanks to the combination of Hoechst 33342, that identifies DNA bearing particles (spermatozoa) and Propidium Iodide (PI) that only stains dead sperm. Changes in membrane permeability are detected with YoPro-1 in combination either with PI or Hoechst allowing to detect spermatozoa in a early stage of membrane destabilization, finally the percentage of spermatozoa depicting active mitochondria are detected using Mitotracker deep red.

Additional markers can be incorporated to these panels, for example specific antiphosphotyrosine antibodies labeled with krome orange or pacific orange and detection in the V2 channel (405/525-50) and/or a calcium yellow sensor (488/585-40) can be incorporated to be detected in the B2 channel to asses sperm capacitation.

This panel only represents an example of the multiple combinations that multiparametric flow cytometry offers. Potential combinations of fluorophores and panels have been recently published (Petrunkina and Harrison, 2013)

Fixable dyes

Recently new fixable dyes have become available in the market (www.lifetechnologies.com). These dyes are available in multiple colors easily allowing multicolor experiments. These assays are based on the reaction of a fluorescent reactive dye with cellular amines. The reactive dye can permeate the compromised membranes of the necrotic cells and react with free amines both in the interior and on the cell's surface resulting in intense fluorescent staining. In contrast only the cell surface amines of the intact cells are available to react with the dye resulting in relatively dim staining.

The discrimination is completely preserved following fixation of the sample by formaldehyde under conditions that inactivate pathogens. Moreover this assays use only one channel of the flow cytometer leaving the other channels available for multicolor panels.

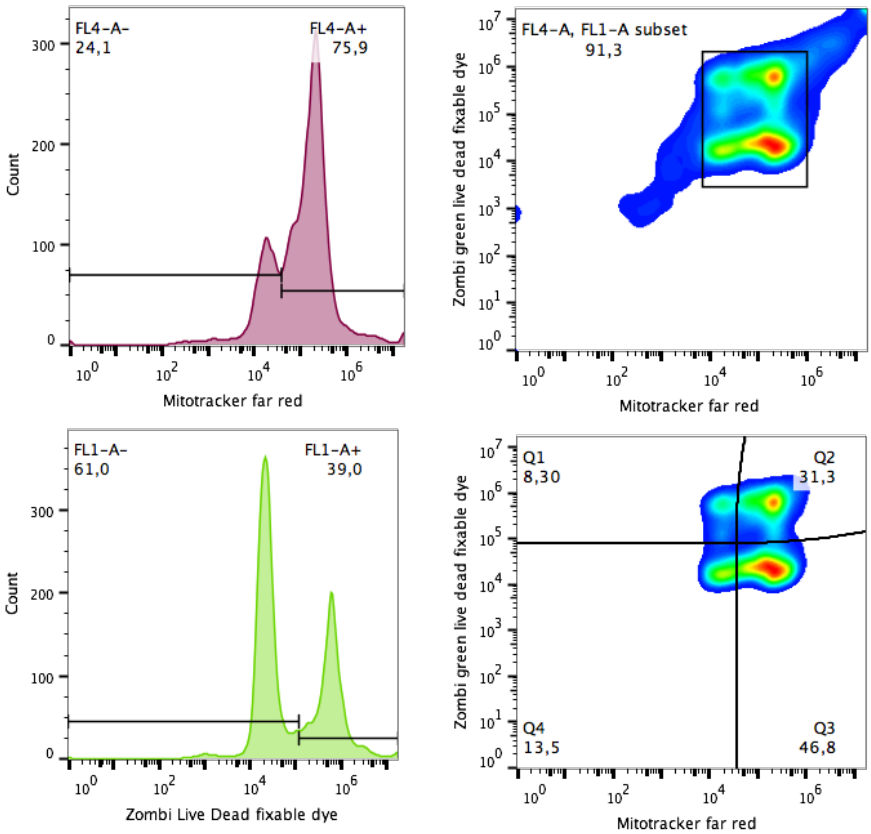

Figure 2. Example of a fixable dye protocol, sperm was stained with Live Dead fixable dye and mitotracker deep red. This assay allows to determine the subpopulation of live spermatozoa, with high mitochondrial membrane potential, represented in Q2.

Cell sorting; not only for sexing?

The theory of the existence of sperm subpopulations within the mammalian ejaculate is gaining growing consensus within the scientific community (Abaigar *et al*., 1999, 2001; Quintero-Moreno *et al*., 2003; Martinez *et al*., 2006). The mammalian ejaculate is a heterogeneous group of different sperm subpopulations showing different responses to physiological or biotechnological stimuli (Abaigar *et al*., 1999; Martinez-Pastor *et al*., 2005), motility patterns (Quintero-Moreno *et al*., 2003, 2004; Ortega-Ferrusola *et al*., 2009c) and even different morphometric characteristics (Thurston *et al*., 2001; Nunez-Martinez *et al*., 2005, 2007; Peña *et al*., 2005a). In many species semen is ejaculated in fractions, where spermatozoa are embedded in the secretions of the male accessory glands, in variable numbers, with variable amounts of fluid and, probably proteins, in each fraction (Rodriguez-Martinez *et al*., 2008). This heterogeneity in

the composition of seminal plasma affects sperm function, as has been demonstrated *in vitro* (Caballero *et al*., 2004; Rodriguez-Martinez *et al*., 2008; Saravia *et al*., 2009). However once the ejaculate is deposited in the female genital tract, the spermatozoa losses contact with the seminal plasma, and are exposed to different environments within the female genitalia. Using the boar species as model, and in experiments performing heterospermic inseminations (Satake *et al*., 2006), the role of the presence of sperm subpopulations in sperm selection has been disclosed. When spermatozoa from two or more boars are mixed and females inseminated the resulting litters are skewed in favor of one male. Bicarbonate responsiveness varies among sperm subpopulations between males, and specific oviductal proteins modulate this response. This mechanism selects sperm subpopulations to reach the oocytes for fertilization. All these scientific evidences provide an explanation of why the overall correlation with fertility is usually low when laboratory test of sperm function that ignore the sperm subpopulation structure are performed (Rodriguez-Martinez, 2003; Holt and Van Look, 2004). One of the potential roles of this sperm subpopulation structure may be a mechanism to help the fertilizing subpopulation of spermatozoa to avoid the immune response of the female while the immune system focus in redundant sperm. Recent research suggests that spermatozoa presenting phosphatidylserine (PS) translocation are preferentially phagocytized; in this way intact spermatozoa have greater possibilities to reach the oocyte for fertilization. Based in the assumption of the existence of a specific fertilizing group of spermatozoa in the ejaculate, recent studies have tried to identify this specific subpopulation and develop techniques for its selection based in flow cytometry (Sousa *et al*., 2011; Ribeiro *et al*., 2013). Sousa *et al*. (2011) used as sorting criteria the spermatozoa showing active mitochondria, and found that this defined a more functional population, with lower chromatin damage and sperm more able to decondense and participate in early embryo development. Furthermore flow cytometry cell sorting was better that traditional swim up for sperm selection. In another experiment Ribeiro *et al.* (2013) used the YoPro-1 staining as sorting criteria, the subpopulation selected was YoPro-1 negative spermatozoa. Using this approach dead and apoptotic spermatozoa were removed, and compared with traditional swim-up techniques the sorted viable population showed a significantly reduced population of spermatozoa with fragmented DNA. Although these techniques may be difficult to implement in production animal practices, may deserve consideration in cases of subfertility in animals of high individual value.

Concluding remarks

Flow cytometry has been in use for sperm analysis from the early 90s, first as a research tool and later introduced in large AI centers; initially most flow cytometers used a single source of excitation (normally a blue laser). More recently more sophisticated equipment has become available in veterinary andrology labs allowing multiple sources of excitation and detection of a large number of parameters. The development of assays that can evaluate simultaneously multiple sperm compartments and functions in a large number of cells shall improve sperm evaluation and establish better correlations with field fertility.

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Status of embryo production in the world

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Abstract

In the last century, agriculture has seen the introduction of innovating reproductive biotechnologies that have permitted this field to grow significantly. In the early 20th century, introduction of semen cryopreservation and artificial insemination has propelled animal agriculture worldwide with the possibility to import and export in a biosecure way genetics from different species. Then, with the development of embryo transfer, it was possible to import and export not only half of the genetic component by disseminating frozen embryos in biosecure manners. Later, the introduction of ultrasonography (which gave us transvaginal ovum pick-up) and *in vitro* fertilization (IVF) revolutionized the speed at which generations of embryos could be produced thus shortening the generation gaps between these important genetics for farmers. Finally, the introduction of genomics again revolutionized the precision and speed at which farmers could identify the desired genetics. The bovine industry is an example of a niche that profited by the development of these technologies. In the last 15 years, IVF embryo production has increased significantly year after year with an all-time high of 42% of the total embryos produced in 2013 were of IVF origin. There are several reasons why IVF is being used more and more in the embryo transfer business: *in vitro* culture media have improved significantly; Introduction of sexed semen for IVF permits farmers to get over 90% of embryos of the desired sex; The interval between generations has reduced significantly with the identification of the next elite male and female genetics using genomic technology. The international agricultural community will benefit by integrating new technologies such as IVF in their operations. It is important that international societies such as SBTE and IETS continue to support scientists and players in this field to develop these technologies.

Keywords: genomics, *in vitro* fertilization, IVF, sexed semen.

Mini-Review

The last 80 years have seen great innovations in the field of Assisted Reproductive Technologies (ARTs). The introduction of innovating ARTs throughout history has rendered farmers and their agricultural businesses more efficient and more profitable. Between the 1930s and 1960s, agriculture has seen the introduction of great innovations such as artificial insemination, semen cryopreservation, oestrous synchronisation and embryo transfer (ET). It is not before the 1980s and 1990s that we witnessed the next generation of biotechnologies that revolutionized the ET world - ultrasonography, embryo freezing and sexing, *in vitro* fertilization (IVF), cloning and semen sexing. Finally, it is in the 21st century that we witnessed yet another innovation that has revolutionized the agricultural business - genomics.

As president of the International Embryo Transfer Society (IETS), I have the opportunity to exchange with many peers in the ET field. IETS's Data Retrieval Committee, chaired by Dr George Perry, consists of a group of individuals from around the world that collect and present world-wide data on activities related to ET technologies in domestic farm animals. The most recent report can be found in the 2015 March IETS Newsletter (Perry, 2015). It is important to note that the data reported in this report is only as valid as the number of countries that have participated. In 2013, only 40 countries have offered embryo data. But the report still gives a good representation of the embryo production status world-wide.

The bovine industry remains the niche that uses extensively ARTs to propagate genetics. Figure 1 illustrates the evolution of both *in vivo* and *in vitro* bovine embryo production between 1997 and 2013. In this figure, it is apparent that *in vivo* embryo production increased significantly between 1997 and 2005. Then *in vivo* embryo production leveled off in 2006 and seemed to decrease slightly with the following years up to 2013. On the other hand, although hardly used in the late 1990s, IVF embryo production has increased significantly year after year with an all-time high of over half a million IVF embryos produced in 2013, which represents 42% of the total embryos produced that year (Fig. 1). It is important to note that in 2013, South America alone produced 73% of the IVF embryos while North America produced 22% of these embryos. Interestingly, of the IVF embryos transferred in North America, approximately 20% of these are frozen, while only 5% of the IVF embryos transferred in South America are frozen.

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There are several reasons why IVF is being used more and more in the last 5 years in the ET business.

In vitro culture media have improved significantly in the last 15 years. The migration from a 1-step system that used serum supplementation and/or cell co-culture to sequential defined or semi-defined media resulted in embryos of higher quality, similar to embryo produced *in vivo*. Older IVF systems resulted in embryos that were less cryotolerant to conventional slow-freezing protocols when compared to *in vivo* embryos (Abe *et al*., 2002) and in calves that exhibited abnormal offspring syndrome (Farin *et al*., 2006). Recent defined IVF media result in embryos that survive slow-freezing protocols making it possible to apply Direct Transfer (DT) techniques just like *in vivo* produced embryos. Because DT of frozen embryos is a technique utilized world-wide, this opens up opportunities to export IVF embryos as easily as *in vivo* embryos. Although the import/export of frozen IVF embryos is still limited, different players, whether from the private sector, the universities, or the government, are working with appropriate regulatory agencies to open these markets to meet the global demand for bovine genetics.

- The introduction of sexed semen for IVF (Garner and Seidel, 2008) use has generated new appeal for this assisted reproductive technology. IVF remains an expensive technology with fixed costs that can result in a higher cost per embryo when compared to conventional *in vivo* flushes. Producing over 90% of female embryos for dairy producers following an IVF cycle provides an added-value that makes IVF appealing even at a higher cost per embryo. The beef market can use male sexed semen and produce over 90% male embryos. Additionally, scientists developed 'Reverse Sorting' which consists in thawing a few straws of conventional non-sexed semen, sex the semen after thawing, and use the freshly sexed semen right away in IVF (Morottia *et al*., 2014). This way, clients can sex almost any frozen semen available on the market.
- Genomics has changed the bovine genetic industry (Shojaei Saadi *et al*., 2014). The interval between generations has reduced significantly with the identification of the next elite male and female genetics using genomic technology. Therefore, the time interval a producer may have to profit from the new genetic he calved on his farm is shorter. So any assisted reproductive technology that can produce many embryos in a short period of time becomes very interesting and profitable. One of IVF's major advantage is that within a 40 to 60 day period, a producer would have time to perform 1 conventional flush *vs*. 4 IVF cycles using superovulated donors. So the gain in higher numbers of embryos per time period becomes significantly advantageous when using IVF.

In conclusion, as scientists worldwide acknowledge the challenges we are faced with feeding the growing global population, the international agricultural community will benefit by integrating new technologies in their operations to assure the sustainability of global livestock and meat demands. IVF seems to be one of these important innovations that will assure the trade of genetics of various species across the globe. It is important that international societies such as SBTE and IETS continue to support scientists and players in this field to develop and promote the different ARTs developed by our peers.

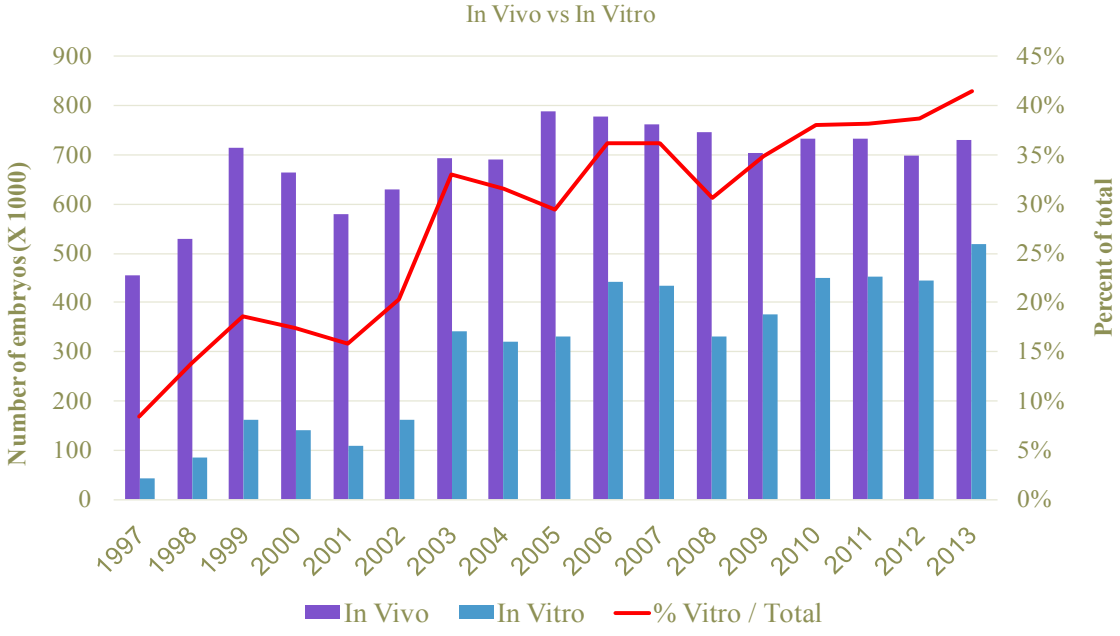

Figure 1. Worldwide *in vivo* and *in vitro* bovine embryo production between 1997 and 2013.

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New aspects of sperm physiology and sperm oocyte interactions

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Abstract

Recent aspects of sperm biology that readily impact on reproductive medicine and biotechnology are briefly overviewed in this paper. The new concept of reactive oxygen species (ROS) homeostasis, and its implications on sperm functions, the heterogeneous nature of the ejaculate and the epigenetic information contained will be overviewed. Additionally, the concept of sperm senescence will be discussed as some aspects of sperm oocyte interaction.

Keywords: CASA, ROS, sperm, subpopulations.

Introduction

In recent years the knowledge of sperm biology has increased exponentially, and new findings in sperm biology are impacting reproductive medicine and technology in animals and humans. In this paper, aspects of sperm biology that have been object of research in the last years in the laboratory of the author will be discussed.

The spermatozoa a redox regulated cell

Reactive oxygen species are byproducts of various metabolic processes, and now are recognized as important regulators of many cellular functions (Stowe and Camara, 2009). The mitochondrion is considered as the major source of reactive oxygen species in most cells. Superoxide ($O_2\bullet^-$) can be generated at different points within the electron transport chain, by univalent reduction of oxygen and spontaneously or enzymatically dismutates to H_2O_2. Most superoxide is converted to H_2O_2 by superoxide dismutases inside and outside of the mitochondrial matrix, and, superoxide in low and controlled amounts exerts important regulatory cellular functions. Excess of H_2O_2 can combine with Fe^{2+} to form reactive ferryl species. In the presence of nitric oxide (NO•), O2•- forms the reactant peroxynitrite (ONOO•), and ONOOH induced nitrosylation of proteins, DNA, and lipids can modify their structure and function (Stowe and Camara, 2009). Numerous studies indicate that ROS are important regulators of sperm function (de Lamirande and Gagnon, 1993, 2002, 2003; Zini *et al*., 1995; de Lamirande *et al*., 1997, de Lamirande and Lamothe, 2009). An important aspect, sometimes neglected, is the type, origin and main role of the different ROS that can be formed during sperm metabolism. Superoxide is short lived ($t_{1/2}$ 1ms) and cell impermeant, while H_2O_2 is more stable and cell permeant. Nitric oxide (NO•) is synthesized through the conversion of l-arginine to l-citruline by nitric oxide synthase (NOS). These enzymes are present in the stallion spermatozoa, possibly as sperm specific isoforms (Ortega Ferrusola *et al*., 2009a). Moreover recent evidence suggests that stallion sperm mitochondria produce significant amounts of NO (Ortega Ferrusola *et al*., 2009a). Nitric oxide has a relatively long half life (1 s) and is more reactive than $O_2\bullet^-$. These compounds are considered, when produced in a controlled manner, as signaling molecules involved in a variety of sperm functions. Other molecules such as the hydroxyl radical (•OH) the peroxynitrite anion ($ONOO^-$) and lipid peroxides are considered more toxic to the spermatozoa and with less regulatory functions. Functions believed to be redox regulated in spermatozoa include activated and hyperactivated motility, chemotaxis, capacitation and the acrosome reaction. Controlled ROS production occurs during capacitation in spermatozoa (Agarwal *et al*., 2014), this controlled production triggers signaling pathways initiated by an increase in cyclic adenosine 3'-5' monophosphate cAMP. Increased cAMP activates protein Kinase A (PKA) and the subsequent phosphorylation of extracellular regulated kinase like proteins and finally tyrosine phosphorylation of proteins in the fibrous sheath of the spermatozoa, leading to sperm hyperactivation. Acrosome reaction and sperm oocyte fusion also depend of ROS activated cellular pathways.

Sperm not only DNA

The sperm genome has to be intact to participate in embryo development. It is, however, susceptible to oxidative DNA damage, so it is important to determine whether it remains intact when semen is manipulated and cryopreserved. In addition, semen also contains a series of small regulatory non-coding RNA (ncRNA) that contain 19 to 22 nucleotides. These microRNA (miRNA) are found in both the seminal plasma and spermatozoa. The miRNA are key of gene expression. They act epigenetically and play an important posttranscriptional modifiers role in the acquisition and maintenance of male fertility. They are

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abundant in bull sperm and show differential expression in relation to fertility levels of different sires. They are delivered to the oocyte at fertilization and modulate the first cleavage divisions (Kleene *et al*., 2010).

Mammalian ejaculate is composed of different sperm subpopulations

Growing consensus exists within the scientific community regarding the existence of sperm subpopulations within the mammalian ejaculate (Abaigar *et al*., 1999). Rather than an homogenous population in which all the spermatozoa shows the same behaviour, the mammalian ejaculate is an heterogeneous group of different sperm subpopulations showing different responses to physiological or biotechnological stimuli, motility patterns and even different morphometric characteristics. Over the years the ejaculate has been considered and analyzed as an entity, and the spermatozoa considered as a single sperm subpopulation. In recent years, the complex nature of the mammalian ejaculate is being unveiled more and more (Abaigar *et al*., 2001; Nunez-Martinez *et al*., 2007). In many species semen is ejaculated in fractions, where spermatozoa are embedded in the secretions of the male accessory glands, in variable numbers, with variable amounts of fluid and, probably proteins, in each fraction (Rodriguez-Martinez *et al*., 2009). This heterogeneity in the composition of seminal plasma may affect greatly sperm function, as has been demonstrated *in vitro*. However once the ejaculate is deposited in the female genital tract, the spermatozoa losses contact with the seminal plasma, and are exposed to different environments within the female reproductive tract. Using the boar species as model, and in experiments performing heterospermic inseminations (Satake *et al*., 2006) the role of the presence of sperm subpopulations in sperm selection has been disclosed. When spermatozoa from two or more boars are mixed and females inseminated the resulting litters are skewed in favour of one male. Bicarbonate responsiveness varies among sperm subpopulations between males, and specific oviductal proteins modulate this response. This mechanism selects sperm subpopulations to reach the oocytes for fertilization. All these scientific evidences provide an explanation of why the overall correlation with fertility is usually low when laboratory test of sperm function that ignore the sperm subpopulation structure are performed.

Kinematic subpopulations

Sperm motility is measured to provide information about the likely fertility of an individual an/or to assess the viability of a semen sample after liquid storage or cryopreservation In these situations motility has to be considered in three ways, first the percentage of cells showing movement, secondly the characteristics of the movement itself and thirdly the speed of the movement. Although sperm motility evaluation is normally one of the first steps of the spermiogramme (Rodriguez-Martinez, 2003), the amount of predictive information given, even using computerized systems, have been scarce and even disappointing. In spite of this, recent evidences suggest that the pitfall in the predictive value of motility relays in the use of the information given and not in the intrinsic value of the motility itself (Holt *et al*., 2007). One of the reasons for the lack of power of conventional parameters is the inherent heterogeneity of mammalian semen; it is one of the most variable of all biological fluids. Sperm motility is the most widely used indicator of sperm quality, however this parameter is dependent of a large number of factors and usually its estimation is largely subjective. In addition sperm motility is evaluated *in vitro*, and what is seen *in vitro* may not reflect at all the behaviour of the spermatozoa during the fertilization process. The fact is that in mammals the significance of sperm motility is a very complex phenomenon, since the spermatozoa are exposed to very different environments, with capability to modify cell signaling pathways, both in the male and female genital tract. In addition, most of the sperm transport process is mediated by the female genital tract, and not by the intrinsic motility of the spermatozoa. During sperm transport, spermatozoa are selected. At coitus billions of spermatozoa are deposited in the female genital tract, but only a few thousands (those of better quality) reach the uterotubal junction. Depending of the species, the spermatozoa may find two main barriers through its journey to reach the oocyte, the cervical barrier (humans, ruminants, canine) and the uterotubal junction in all species. It is clear that only a minority of the spermatozoa present in the ejaculate is retained in the female reproductive tract. In humans it has been estimated that only less than 1% of the spermatozoa are retained in the female reproductive tract and that this supports the notion that only a minority of sperm enters the cervical mucus and ascends higher in the female reproductive tract (Suarez and Pacey, 2006). The uterotubal junction acts as a selective barrier, allowing only the "best spermatozoa" reaching the sperm reservoir (Liu *et al.*, 1991). In this regard, high sperm velocities appear to be a critical factor to colonize the oviduct (Liu *et al*., 2010). The fact is that when sperm motility is evaluated using traditional approaches, a number of important factors are ignored: a) that not all the spermatozoa in the semen sample will reach the oviduct; b) that sperm motility will be displayed in different manner in response to different environments within the female genital tract; c) that sperm velocities are the key factors to colonize the oviduct, and d) that the sample is a mix of different sperm subpopulations with different ability to response to signals arising in the female genital tract. However the analysis of the sperm subpopulation structure, using sperm velocities as

variable to disclose the heterogeneity of the ejaculate has proven to be more informative to asses the quality of a given semen sample.

Morphometric subpopulations

Most studies on sperm subpopulations have been performed using kinematic parameters, but some research has been done using computerized morphometry to disclose sperm subpopulations. In the laboratories of the authors, the sperm morphometric subpopulation structure has been studied using the canine, equine and porcine species as models. Also the sperm subpopulation structure of wild ruminants has been described. Studies in boars, using sequentially principal component analysis (PCA), clustering, and discriminant analyses have been developed to identify sperm morphometric subpopulations in well-defined portions of the fresh boar ejaculate. In a recent study using the boar as a model (Saravia *et al*., 2007) within morphologically normal spermatozoa, different sperm subpopulations were disclosed based in head size and other morphological parameters, such as the regularity of sperm head shape.

Spermatozoa are present in the ejaculate in different physiological status

The spermatozoa are eliminated at ejaculation embedded in the secretions of the male accessory glands. In species such as pig, horses and dogs, the ejaculate is eliminated in different jets, accompanied of different subsets of secretions from the accessory glands. Whether or not this modifies sperm physiology is still to be fully determined, but the fact is that not all the spermatozoa within a given ejaculate are in the same physiological status. Studies in pigs demonstrate that the sperm present in the first 10 ml of the sperm rich fraction have different motility patterns, capacitation status, and even different size. Also these spermatozoa are the first to colonize the sperm reservoir at the oviduct. Studies in horses demonstrate that a significant subset of spermatozoa present in a given ejaculate is activated to experience an apoptotic process shortly after ejaculation (Ortega Ferrusola *et al*., 2008). These spermatozoa are unlikely to be able to fertilize an oocyte. So, a physiological explanation for these phenomena is warranted. The fate of most of the spermatozoa from a given ejaculate is to be removed from the female genital tract, either due to semen reflux after breeding, either being phagocytosed within the female uterus. Only a few thousand spermatozoa colonize the sperm reservoir at the utero- tubal junction. This means that the loose of spermatozoa by reflux and phagocytosis is a part of a process of sperm selection. It is believed that the heterogeneous nature of the ejaculate is involved in this sperm selection. In fact, although to be demonstrated in all the species, spermatozoa depicting PS externalization are preferentially phagocytosed in humans. Spermatozoa depicting PS externalization can be considered as prematurely activated, probably acting as a barrier against the female macrophages, thus protecting other spermatozoa. In the porcine species the proportion of spermatozoa with PS externalization varies between ejaculate fractions (Peña *et al*., 2003). In stallions, fresh sperm present a high percentage of spermatozoa showing caspase activity, both pro-caspases and active caspases 3, 7 and 9 have been detected in fresh samples (Ortega Ferrusola *et al*., 2008). From all these findings is clear that the spermatozoa present in a given ejaculate are in different physiological states at ejaculation, and this heterogeneity may be related to sperm selection within the female reproductive tract.

Born to dye; only one last. Do mitochondria control the lifespan of ejaculated spermatozoa?

Although sperm death after ejaculation is due to ATP depletion, other forms of sperm demise are described. Both “apoptotic like events” (Aitken *et al*., 2012a, b; Gallardo Bolanos *et al*., 2014), and an “autophagy like” mechanism may also be involved in sperm death after ejaculation (Gallardo Bolanos *et al*., 2012; Bolanos *et al*., 2014). More interestingly, different subpopulations of spermatozoa are in more advanced stages of senescence and may die at different intervals after ejaculation (Auger *et al*., 1993; Barroso *et al*., 2006; Gallon *et al*., 2006). Many spermatozoa with apoptotic changes appear in the ejaculate, and apoptotic changes including phosphatidylserine (PS) translocation (Peña *et al*., 2003; Brum *et al*., 2008; Martin *et al*., 2004), increase in membrane permeability (Ortega Ferrusola *et al*., 2009a), low mitochondrial membrane potential (da Silva *et al*., 2011; Aitken *et al*., 2012a; Garcia *et al*., 2012), activated caspases (Caselles *et al*., 2014; Gallardo Bolanos *et al*., 2014) and DNA fragmentation (Smith *et al*., 2013; Gillan *et al*., 2005) have been characterized in the ejaculate. Although the significance of these changes is still under debate, some points of consensus are being achieved. Perhaps, the most important aspect is that all spermatozoa are programmed to die, and that only one sperm reaches immortality through fertilization (Aitken and Koppers, 2011; Aitken *et al*., 2012a). Importantly many sperm biotechnologies accelerate this pathway to sperm death (Ortega-Ferrusola *et al*., 2008; Balao da Silva *et al*., 2013; Peña *et al*., 2011; Petyim *et al*., 2014). This form of sperm death appears to be dependent on the activation of an intrinsic apoptotic cascade originated in the mitochondria after unbalanced mitochondrial ROS generation (Aitken and Curry, 2011; Koppers *et al*., 2008). This later may occur after exhaustion of intracellular antioxidant defenses, particularly intracellular glutathione. Although this mechanism has been primarily described in humans, evidences suggest

that a similar scenario occurs in stallions, mainly during processes of conservation of sperm (Ortega-Ferrusola *et al*., 2008, 2009b). The maintenance of sperm viability depends on the phosphorylation status of specific pro-survival proteins. One of this, Akt is activated through phosphorylation at threonine-308 or serine-473 (Alessi *et al*., 1997; Yu *et al*., 2005). After phosphorylation, Akt functions through phosphorylation and inhibition of Bad (serine-136) or caspase-9 (Cardone *et al*., 1998). Bad is a proapoptotic member of the Bcl family that promotes cell death by dimerization with Bcl-2 or Bcl-X_L (Yang *et al*., 1995). Bad phosphorylation at four different serine residues (serine-112, -136, -155, or -170) has been characterized as inactivating Bad (Datta *et al*., 1997; Lizcano *et al*., 2000; Dramsi *et al*., 2002; Danial *et al*., 2003). Provided that sperm Akt is phosphorylated at Ser473 and/or Thr308 sperm motility and integrity are maintained; with de-phosphorylation of Akt, caspases are activated and motility is rapidly lost (Gallardo Bolanos *et al*., 2014), a similar mechanism occur in human sperm (Koppers *et al*., 2011). It appears that de-phosphorylation of Akt depends of different factors such as ATP depletion, unbalanced ROS and removal of pro-survival factors (Pujianto *et al*., 2010). On the contrary, dead receptor mediated sperm death may also occur. Some evidences indicate the sperm death can be triggered through the activation of toll like receptors by bacteria (Das *et al*., 2011; Fujita *et al*., 2011), ROS released by death sperm (Roca *et al*., 2013), and TNFα or other pro death factors released by other spermatozoa activating extrinsic apoptotic pathways (Macias Garcia *et al*., 2012; Mendoza *et al*., 2013).

Fertilization: the miracle of life

For fertilization to occur in mammals, ejaculated spermatozoa must reach the egg, that after ovulation has move from the ovary to the fallopian tube. Very few of the ejaculated spermatozoa, less than 1 million in humans reach the fallopian tube, moreover the spermatozoa undergo capacitation before being able to penetrate the cumulus layer, bind to the sperm receptor of the egg coat, and undergo the acrosome reaction that allows sperm penetration through the egg coat and fusion with the egg. The percentage of capacitated spermatozoa is low (around 10% in humans), so the number of spermatozoa that can reach and fertilize the egg is small. Due to this slow number of capacitated sperm, mechanisms of sperm guidance are crucial for successful fertilization to occur. Two active mechanisms of sperm guidance have been described in mammals, chemotaxis and thermotaxis (Eisenbach and Giojalas, 2006). Only the small fraction of capacitated sperm is chemotactically active. The spermatozoa use both active swimming and passive drag by female genital tract muscular contraction to reach the storage site at the oviduct. Once here, a small percentage of spermatozoa undergoes capacitation; capacitated sperm reach the egg guided by a combination of chemotaxis, termothaxis and, perhaps oviductal contractions. Chemoattractants are present in the oviductal fluid and are also secreted by the egg and surrounding cumulus cells. Initially capacitated spermatozoa at the storage site, use an ovulation-dependent temperature gradient between this site and the fertilization site (Eisenbach and Giojalas, 2006). As spermatozoa approach the fertilization site they probably sense a chemoatractant gradient that guides them to the egg. Confirmed chemoatractants for mammalian spermatozoa are atrial natriuretic peptide, bourgeonal, lyral, small peptides from the follicular fluid (1.3 to 13 kDa), progesterone and rantes (Eisenbach and Giojalas, 2006).

The fertilization site in the oviduct has been shown to be 1-2ºC warmer that the storage site for spermatozoa in rabbits and pigs, this change is due more to a temperature decrease in the storage site than increase at the fertilization site (Eisenbach and Giojalas, 2006).

Fertilization triggers a complex cellular programthat transforms two highly specialized meiotic germ cells, the oocyte and the sperm, into a totipotent embryo. Sperm cells initially bind to the zona pellucida of the egg made of just a few glycoproteins, zona pellucida binding protein 1 (ZP1) to ZP4 in humans (Clift and Schuh, 2013). Early studies identified ZP3 as a potential primary sperm receptor, however now is considered that ZP3 alone is not sufficient for sperm binding. It is possible that ZP proteins together adopt a three dimensional structure that presents a binding site for sperm. This binding site is lost after fertilization preventing polyspermy. The protease responsible for ZP2 cleavage has been identified, and is termed ovastacin. ZP proteins are modified with oligosaccharides at Asp (N-linked) and/or Thr (O-linked resiues). Most of them terminate with the sialyl Lewis X tetrasaccharide motif, that plays a major role in sperm-egg binding (Clift and Schuh, 2013). Upon binding to the egg the sperm experiences the acrosome reaction with release of hydrolytic and proteolytic enzymes that opens a way for the fusion of the spermatozoa with the plasma membrane of the egg. The fusion of the sperm with the egg triggers the completion of the second meiotic division and the transition to mitosis. The sperm induces rise in free Ca2+ in the egg, in mammals a series of Ca2+ oscillations triggers a temporally ordered sequence of events, including the release of cortical granules, completion of the second meiotic division, translation of maternal mRNAs, and ultimately the transition of meiosis to mitosis. Increase of Ca2+ is triggered by phospholipase Cζ (PLCζ) that is introduced in the egg by the sperm. PLCζstimulates the production of inositol 1-4-5 trisphosphate, which binds to receptors in the ER causing the release of Ca2+, and triggering the exit from meiosis. Eggs arrest in metaphase of the second of the second meiotic division,

while they await for fertilization. This arrest is mediated by the activity of cytostatic factor (CSF). An essential mediator of CSF activity is EMI2 (early mitotic inhibitor 2). EMI2 maintains metaphase II arrest by inhibiting the anaphase-promoting complex, which targets cyclin B and the separase inhibitor securin for degradation (Clift and Schuh, 2013). The increase in intracellular Ca^{2+} levels upon fertilization activates Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which phosphorylates EMI2. EMI2 is further phosphorylated by Polo-like kinase 1 (PLK1) and subsequently targeted for degradation by the SCF (SKP2-cullin 1-F-box protein) ubiquitin ligase. This leads to the activation of the APC/C, destruction of cyclin B and securin, elimination of half of the sister chromatids into the second polar body and the formation of the female pronucleus (Clift and Schuh, 2013). Almost immediately after fertilization, the increase in intracellular Ca^{2+} levels triggers exit from meiosis and the formation of a female pronucleus, while the sperm genome undergoes decompaction. Protamines are rapidly removed from the sperm pronucleus, and the DNA is re-wrapped around nucleosomes that contain the histone H3 variant H3.3, which is replaced with canonical histone H3 during DNA replication. Although now equivalently structured in nucleosomes, the two pronuclei retain some parent-specific histone methylation patterns, particularly at pericentromeric heterochromatin regions that are equilibrated gradually during the first embryonic divisions. For the zygote to acquire totipotency, the parental genomes must also undergo extensive epigenetic reprogramming, which involves global DNA demethylation. The sperm genome is highly methylated compared with that of the egg. Within hours of fertilization, however, the sperm pronucleus undergoes rapid active demethylation before DNA replication.

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Snooping on a private conversation between the oviduct and gametes/embryos

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Abstract

After a long journey travelling up the maternal tract the spermatozoa will meet the oocyte. As a result, an early embryo will promptly commence its development while travelling down the oviduct. These short but vital journeys of gametes and embryos are accompanied by important changes in the maternal tract. In particular, from the oviduct, which provides an optimal environment for gamete maturation and transport, fertilization and early embryo development. In fact, to achieve a successful pregnancy the oviduct should keep a fruitful dialogue with the gametes followed by an appropriate communication with the embryo(s). In the present review, the transcriptomic and proteomic changes induced by gametes and embryos in the oviduct as a result of this early dialogue will be reported. A special mention of the differential conversation between the oviduct and X and Y-chromosome-bearing spermatozoa, which might be at the basis of gender selection, will be provided. Subsequently, the ability of the embryo to modulate its own oviductal environment thus avoiding its maternal rejection will be discussed. Ultimately, a third player will be introduced in this dialogue, exosomes/microvesicles, which have been proposed as early mediators of these maternal-gamete/embryo interactions. Snooping on the private conversation between the oviduct and gametes/embryo may provide some molecular clues about the mechanisms that mediate these interactions. Moreover, knowing the genes and proteins that pilot the success of the early reproductive events will offer great opportunities for the improvement of assisted reproductive technologies and animal breeding efficiency.

Keywords: embryos, exosomes, gametes, oviduct interactions.

Introduction

In mammals, maternal interactions with gametes and embryos are the basis for the success of any reproductive event. The oviduct, or Fallopian tube, which is the maternal tube connecting the ovary and the uterus, plays a vital role in these interactions. It holds the maternal dialogue with gametes and early embryos and provides an optimal environment for gamete maturation and transport, fertilization and early development of the embryo (Hunter, 2005).

The oviduct can be seen as a bidirectional route, where the spermatozoa travel up to meet the oocyte while the early embryo travels down towards the uterus. In most mammals it is divided anatomically into three parts: 1) the utero tubal junction, that connects the oviduct to the uterus; 2) the isthmus, the region associated with the storage of spermatozoa before ovulation and where spermatozoa bind to the oviduct epithelial cells (OEC) on their way to meet the oocyte and; 3) the ampulla, where fertilization takes place. Spermatozoa from most mammals can reside in the oviduct from a few hours up to a maximum of 5-7 days (Holt and Fazeli, 2010). Bats are exceptional among mammals having the ability to store spermatozoa for several months in the uterus or oviducts during hibernation (Bernard and Cumming, 1997). By contrast, the embryo spends only a few days (2-5) in the oviduct, which also varies depending on the species: in mouse 2-3 days (Rafferty, 1970); in pigs 1-3 days (Pomeroy, 1955; Oxenreider and Day, 1965); in cows 2-4 days (Hamilton and Laing, 1946; Crisman *et al*.,1980); in sheep 2-3 (Holst, 1974) and in mares 5-6 days (Freeman *et al*., 1991). To adapt to these different scenarios, the oviduct is spatially and temporally regulated by hormones and also by its interactions with gametes and embryos (Fig. 1).

However, modulation of the oviduct by gametes and embryos is poorly understood. Focusing on these interactions is also a matter of two sides. On one side, there is a modulatory effect of OEC on spermatozoa (Ellington *et al*., 1991) and the oviductal secretions on embryo development (Gandolfi, 1989). On the other side, spermatozoa and the embryo can also modulate the gene and protein expression of the oviduct (Ellington *et al*., 1993; Thomas *et al*., 1995; Fazeli *et al*., 2004; Georgiou *et al*., 2005, 2007; Almiñana *et al*., 2012; Schmaltz-Panneau *et al*., 2014; Yeste *et al*., 2014). Emerging studies are suggesting a third player in these interactions, exosomes/microvesicles, which could act as mediators in the two-way communication system that takes place in the maternal tract (Ng *et al*., 2013; Burns *et al*., 2014).

For simplicity, this review will focus on oviduct-gamete/embryo interactions in mammals. The role of gametes and embryos as modulators of the maternal tract will be addressed in the following pages. In recent years an increasing number of publications have examined this side of the maternal interactions,

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which reflects the importance of these interactions. Snooping on the private conversation between the oviduct, gametes and embryos may reveal the mechanisms that mediate these interactions. Understanding this complex dialogue will shed some light into infertility problems, reduce early pregnancy loss and may even identify the factors that influence the development of the offspring into adulthood.

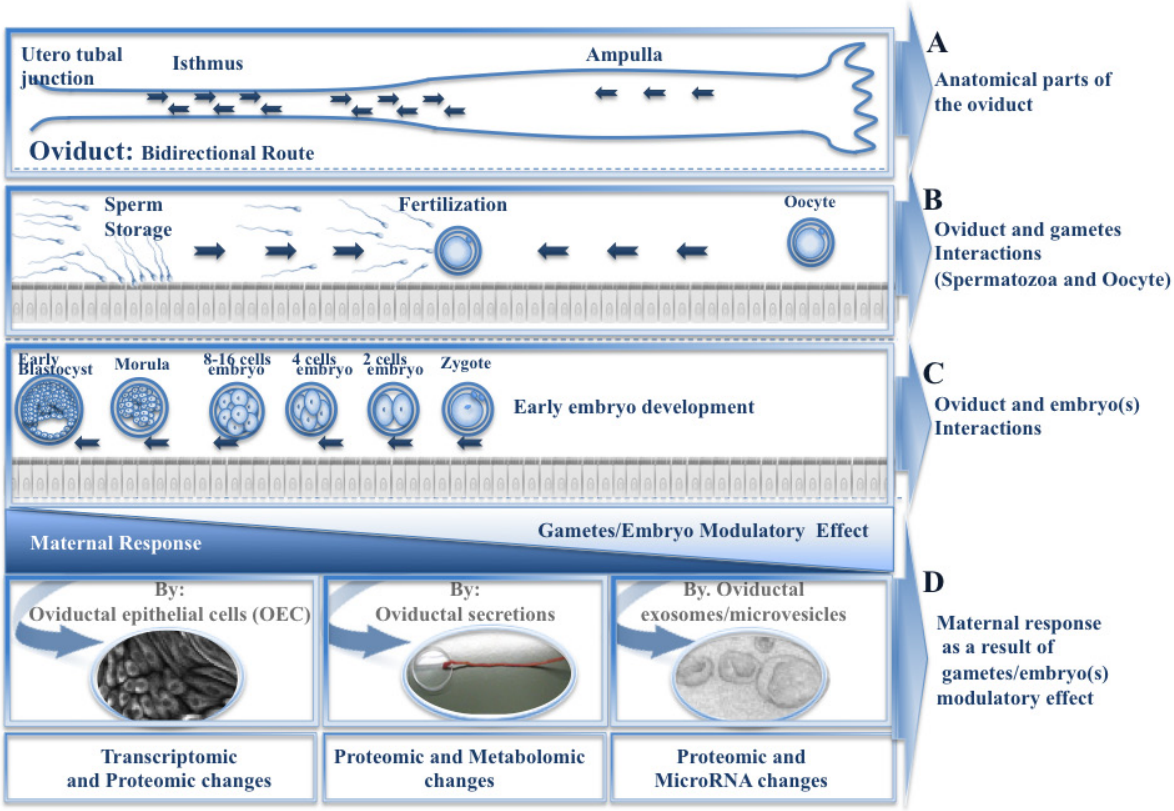

Figure 1. Diagram of anatomic parts of the oviduct and gametes/embryos interactions with the oviduct.

Oviduct and gametes interactions: conversations or negotiations?

The interactions between the oviduct and the gametes involve close and specific contact between them (Hunter and Nichol, 1983; Fazeli *et al*., 1999, 2003). As a result of this contact, a confidential dialogue between the OEC and the gametes takes place. Solid evidence allows us to state that this dialogue is not univocal, and must be seen as a two-way communication system that ensures the success of early reproductive events. On one side, the oviduct and its secretions influence the physiology of the gametes (Avilés *et al*., 2010). On the other side, gametes also modulate the oviductal environment (Fazeli *et al*., 2004; Georgiou *et al*., 2007).

There is no doubt of the vital role of the oviduct in the preparation of male and female gametes for their successful meeting (Coy *el al*., 2012; Avilés *et al*., 2015) but less extensive and detailed research exists on the ability of the gametes to modulate their own oviductal environment. Initial evidence about the way that spermatozoa control the oviductal environment revealed that the attachment of sperm cells to the bovine OEC during co-culture changed the types and quantities of proteins secreted into the conditioned medium (Ellington *et al*., 1993). Several studies using mouse or pig models have further demonstrated a maternal response to spermatozoa (Fazeli *et al*., 2004; Georgiou *et al*., 2005, 2007). Fazeli *et al*. (2004) revealed that the arrival of spermatozoa into the oviduct after mating resulted in alterations of the oviductal transcriptome. Those same alterations were not found when infertile mice, which produce seminal plasma but no spermatozoa (T145H mutant mice), were used in the experiment (Fazeli *et al*., 2004). Georgiou and co-workers showed that the presence of both gametes, spermatozoa and oocytes, altered the oviductal secretory profile (Georgiou *et al*., 2005, 2007). The oviductal response to spermatozoa was different from that induced by oocytes. Spermatozoa induced a specific oviductal proteomic response, modulating the expression of 20 proteins while only one protein was regulated by oocytes. Recently, Artemenko and colleagues using a refined mass-spectrometry-based approach reported an immediate response of the surface proteome of oviductal cells to spermatozoa, which was modulated over time (Artemenko *et al*., 2015). Thirty-

one cell surface proteins were found pronouncedly altered (≥2 fold change) immediately, 1 and 2 h after insemination compared to control. Functional analysis showed that those proteins were associated to structural reorganization of the oviductal epithelium cell surface. Interestingly, oviduct specific glycoprotein (OVGP), a crucial protein in fertilization processes (Buhi, 2002), was strongly increased at the cell surface 1 h after insemination. OVGP was also found up-regulated in response to spermatozoa in sow oviducts (Georgiou *et al*., 2007) but at 24 h after artificial insemination. These findings support the view that the complex transcriptomic and proteomics changes that occur in the oviduct are finely tuned through the dialogue between the oviduct and gametes.

Moreover, the sperm-oviductal dialogue could be at the basis of the intriguing selection of X or Y-chromosome bearing spermatozoa by the oviduct prior to fertilization. Sex allocation of offspring in mammals is usually considered as a matter of chance, being dependent on whether an X- or a Y-chromosome-bearing spermatozoon reaches the oocyte first. Evidence from the field and laboratory suggests that female mammals can bias the sex ratio of their offspring (Clutton-Brock and Lason, 1986; James, 2009). However, no biological mechanism(s) explaining this selection has yet been discovered. A recent study in pigs (Almiñana *et al*., 2014) provided an important mechanistic insight into this phenomenon. By introducing X- or Y-sperm populations into the two separate oviducts of single female pigs using bilateral laparoscopic insemination, Almiñana and co-workers found that the spermatozoa did indeed elicit sex-specific transcriptomic responses. Microarray analysis revealed that 501 from 24123 probes were consistently altered ($P < 0.05$) in the oviduct in the presence of Y-chromosome-bearing spermatozoa compared to the presence of X-chromosome-bearing spermatozoa. From these 501 transcripts, 271 transcripts (54.1%) were down-regulated and 230 transcripts (45.9%) were up-regulated when the Y- chromosome-bearing spermatozoa were present in the oviduct. Two fascinating ideas derived from our study: 1) spermatozoa carrying the Y- or X-chromosome can modulate the oviductal response by activating specific signalling pathways in a gender specific manner and 2) the female reproductive tract can sense the presence of X- or Y-chromosome-bearing spermatozoa in the oviduct before fertilization occurs. The fact that mothers can recognize the difference between X- and Y- bearing spermatozoon is a first prerequisite to allow only one preferred type of spermatozoa to reach the oocyte. Therefore, these sperm-oviduct interactions could be seen more as fruitful "negotiations" if, as a result, one type of spermatozoon might be selected. Although the precise mechanism that might bias the gender selection is not yet elucidated, the study by Almiñana *et al*. (2014) provides candidate genes that might be responsible of this gender selection.

After digging in X and Y-sperm features that could be read by the oviduct and might be involved in the sperm sex-selection, different topographic characteristics on the head of X- and Y-spermatozoa were observed by atomic force microscopy (Carvalho *et al*., 2013). In a similar way, differentially expressed proteins found between bull X- and Y-spermatozoa (Chen *et al*., 2012), might be sensed by the oviduct and help in the sex-selection. Furthermore, emerging studies on the microRNA population of spermatozoa suggest that they could be important players in these sperm-oviductal interactions. MicroRNAs are powerful regulators of gene and protein expression (Bartel, 2004; He and Hannon, 2004) and thus, sperm microRNA could modulate oviductal gene expression. The emerging new ways of embryo-to-embryo communication proposed by microRNA release via exosomes during *in vitro* culture (Saadeldin *et al*., 2014) could be also used by sperm microRNA to interact with the oviduct. To date, only differences in sperm microRNA between fertile and infertile spermatozoa (Lian *et al*., 2009: Abu-Halima *et al*., 2013) and, a potential role of sperm microRNA as chemoattractant-activated transduction signalling and their association to vesicles have been demonstrated (Das *et al*., 2013). But together, such evidence supports the view of microRNAs as "hot" candidates in gender-selection.

Oviduct and embryo(s) dialogue: what does the embryo say to the mother?

The oviduct also plays a direct role in supporting early embryonic development (Gandolfi *et al*., 1989). It provides the best environment for the embryo, matching its requirements, within the short but very vital period before entering the uterus (Besenfelder *et al*., 2012).

Previously, we have mentioned that the arrival of spermatozoa in the oviduct and their binding to oviductal cells initiates a sperm-oviduct signalling dialogue. By contrast following fertilization, the resulting embryo spends the next few days in the oviduct while it is "free-floating" in the maternal tract, and has no direct contact with the mother while travelling down the oviduct to reach the uterus (Hunter, 1980). Because of this, the embryo has been considered relatively autonomous during this early time of its life. The fact that embryos can be routinely produced and developed up to the blastocyst stage *in vitro,* due to the great advancement of reproductive biotechnologies, has reinforced this idea. All together, these facts have encouraged into the view that the oviduct is merely a passive tube for the transport of the embryo on its way to the uterus (Marston *et al*., 1977), rather than an essential organ that offers protection and nutrition for the normal embryo development. However, evidence demonstrating the superior competence of the *in vivo*

embryos compared to the *in vitro* embryos (Rizos *et al*., 2008, 2010; Van Soom *et al*., 2014) and the epigenetic effects of the *in vitro* culture on the embryo developmental potential (Hou *et al*., 2007; Reis e Silva *et al*., 2012; Beaujean, 2014; Bertoldo *et al*., 2014) has made researchers rethink the undoubted role of the oviduct hosting the early developing embryos.

The early developing embryo undergoes a highly orchestrated series of events, such as the first mitotic cells divisions and genome activation. To encompass these early developmental events and allow the delivery of a competent conceptus to the endometrium, the oviductal lining is subjected to dynamic changes (Besenfelder *et al*., 2012). In this regard, researchers have examined the possibility that the embryo could act as a mediator of its own environment (Almiñana *et al*., 2012). However, the complex signals exchanged between the oviduct and the embryos that lead to alterations of the environment in response to embryo(s) are not yet fully understood.

Given the ethical and scientific obstacles associated with *in vivo* embryo-maternal studies, primary OEC cultures have been thoroughly used to study these early embryo-oviductal interactions. Using this model researches have confirmed the existence of a real dialogue between the early embryo and the oviduct (Cordova *et al*., 2014; Schmaltz-Panneau *et al*., 2014). Co-incubation of bovine OEC (BOEC) with bovine embryos induced changes in embryonic gene expression (Cordova *et al*., 2014). Moreover, BOEC from isthmus and ampullar regions increased cleavage rate and blastocyst rate over the control, with BOEC from the isthmus being more capable of supporting early embryo development than BOEC from the ampulla. In response, the embryo was also capable of modifying BOEC gene expression and protein secretion (Schmaltz-Panneau *et al*., 2014). In this regard, thirty-three genes were over-expressed in BOEC in the presence of embryos compared to the control counterpart. Only one gene was down-regulated. Most of the up-regulated genes corresponded to genes regulated or involved in interferon type I signalling pathway. A large number of these interferon tau (IFNT)-induced genes were also found in transcriptional profiling experiments in the bovine uterus (Bauersachs, 2006; Klein *et al*., 2006; Mansouri-Attia *et al*., 2009; Forde *et al*., 2011, 2012). These uterine changes have been mainly associated to pregnancy recognition signals in response to the secretion of IFNT by the conceptus. However, IFNT secretion by bovine embryo starts around 15-16 days after fertilization when the embryo is in the uterus (Bazer *et al*., 1997). Therefore it has been hypothesized that embryonic IFNT could play a key role in maternal pregnancy recognition in the oviduct and in the uterus by activating a set of specific genes before and at the implantation period (Schmaltz-Panneau *et al*., 2014).

Even though BOEC-embryo *in vitro* model studies have proved the existence of certain embryo-oviductal interactions, the question that arises is how far are these *in vitro* interactions from those that occur *in vivo* during early pregnancy. To date, only a few studies have provided evidence of the *in vivo* maternal-embryo interactions in the oviduct at the very early stages of embryo development (Lee *et al*., 2002; Almiñana *et al*., 2012; Maillo *et al*., 2015). Lee *et al*. (2002) compared the gene expression pattern of mouse oviducts containing early embryos and oviduct containing oocytes. The presence of embryos altered the transcriptome profile of the oviduct compared to oocytes. Using a pig model Almiñana and co-workers showed that the changes observed in the oviductal gene expression were dependent on the embryo developmental stage (Almiñana *et al*., 2012), demonstrating a more specific response of the oviduct towards the embryo. Additionally, these authors observed that when the embryo migrated from the oviduct to the uterine horn, the mRNA levels of a selected transcript related to immunity (TICAM2) was down-regulated in both the oviduct and the uterine horn samples. The uterine down-regulation of the immune related genes while the embryo is still in the oviduct might function as in preparing the uterus to accept the embryo.

In a more holistic study of the oviductal changes, Maillo *et al*. (2015) have demonstrated that the early bovine embryo elicits an oviductal response during its transit through the oviduct that may contribute to its subsequent development. Although these authors have used a non-physiological model to prove this dialogue by transferring 50 embryos into the oviduct of a cow, the presence of multiple embryos in the oviduct induced differential transcriptional changes in OEC when compared to the gene expression responses to oocytes. Furthermore, Maillo *et al*. (2015) observed that the presence of multiple embryos in the cow oviduct down-regulated the maternal immune system, confirming previous results obtained by Almiñana *et al*. (2012). Taken together, these studies demonstrated that as a result of the early embryo maternal dialogue the embryo mediates its own environment in the maternal tract. Furthermore, the embryo seems to contribute to its maternal tolerance by modulating the maternal immune system.

On the other hand, the transcriptomic changes observed in the oviduct in response to the presence of the embryo (Lee *et al*., 2002; Almiñana *et al*., 2012; Maillo *et al*., 2015), may be possibly associated with changes in the oviductal secretions at the very early stages of pregnancy. Therefore, it seems imperative to investigate the temporal and spatial secretions triggered by the embryos while they are free floating in the oviduct. So far, much emphasis has been paid to the uterine fluid surrounding the blastocyst or early conceptus (Muñoz *et al*., 2012; Gomez *et al*., 2013; Forde *et al*., 2014), even though early embryonic mortality might occur before embryo reaches the uterus.

Exosomes/microvesicles: mediators of gamete/embryo interactions

Exosomes are small (30-100 nm) membrane vesicles of endocytotic origin that have been identified *in vivo* in all body fluids including follicular (da Silveira *et al*., 2012; Sohel *et al*., 2013), uterine (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) and oviductal fluids (Al-Dossary *et al*., 2013) and can be secreted by most cell types *in vitro*. They specifically carry proteins, lipids, and genetic materials such as DNA, RNA, and microRNA that could be transferred to recipient cells, and may induce epigenetic changes. Exosomes together with microvesicles (bigger vesicles around 50-1000 nm with similar content; Dragovic *et al*., 2011; Gyo¨rgy *et al*., 2011; Turiák *et al*., 2011; Braicu *et al*., 2015) play fundamental biological roles in the regulation of physiological as well as pathological processes, which make them interesting therapeutic vectors (Suntres *et al*., 2013).

Recent studies indicate that exosomes/microvesicles could act as intercellular vehicles in the embryo-maternal dialogue in the uterus (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) and might also mediate the maternal-gametes/embryo interactions in the oviduct. Oviductosomes (Al-Dossary *et al*., 2013) and uterosomes (Ng *et al*., 2013; Burns *et al*., 2014; Ruiz-Gonzalez *et al*., 2015) have been identified recently, but it is still a mystery how they are taken up by gametes and embryos and whether they modulate the maternal interactions to promote successful pregnancy. On the embryo side, only one recent study has shown that *in vitro* produced embryos can secrete exosomes as a possible way of communication among them (Saadeldin *et al*., 2014).

As mentioned above, OEC from primary *in vitro* culture have been thoroughly used as *in vitro* models to study oviduct-embryo interactions in different species and therefore, could be the model of choice to study the role of the exosomes in this unique communication system. However, knowing the large differences between *in vivo* and *in vitro* embryos in terms of embryo quality and gene expression and the different morphologic characteristics and protein expression of OEC from *in vivo* and *in vitro* origin (Rottmayer *et al*., 2006), our laboratory began to characterize the bovine oviductal exosomes from both *in vivo* and *in vitro* origin (Almiñana *et al*., 2015). For this purpose, exosomes secreted by OEC *in vivo* in the oviductal fluid and by OEC *in vitro* in the conditioned media after OEC primary culture were collected by serial ultracentrifugation. Preliminary results by dynamic light scattering analysis revealed different size distribution profiles compatible with exosomes and microvesicle populations from *in vivo* preparations and mostly microvesicle populations from *in vitro* preparations. Protein profile analysis by SDS-PAGE showed quantitative and qualitative differences among the exosomes samples, their cells of origin and the milieu (conditioned media or flushing). In addition, exosomes of *in vivo* and *in vitro* origin exhibited distinct proteomic profiles. Western blot analysis demonstrated that (i) both types of exosomal protein samples were positive for HSP70, a known exosomal protein (ii) *in vivo* exosomes expressed OVGP and heat shock protein A8 (HSPA8), oviductal proteins with known roles in fertilization and early pregnancy. However, only HSPA8 was detected in *in vitro* exosomes. These results have contributed to the first characterization of oviductal exosomes of *in vivo* and *in vitro* origin. In depth analysis of the content of these vesicles will bring new insights into the embryo-oviductal dialogue and will increase our knowledge of the oviductal environment that supports the early stages of embryo development.

In addition, further studies aimed at understanding the molecular mechanisms by which exosomes/microvesicles are internalized by cells may contribute to their therapeutic applications. Mechanisms involving membrane fusion or endocytosis (Del Conde *et al*., 2005; Parolini *et al*., 2009) have been proposed, but it is still unclear whether these vesicles could use more than one route or whether the vesicular uptake is cell type specific (Feng *et al*., 2010). To date, it is known that oocytes can take up exosomes from the follicular fluid, showing a cell-to-cell communication system during oocyte growth (da Silveira *et al*., 2012; Sohel *et al*., 2013). In addition, it has been shown that sperm can take up a Ca2+ regulatory protein, PMCA4, from oviductosomes (Al-Dossary *et al*., 2013). PMCA4 is involved in the capacitation and acrosome reaction, suggesting than oviductosomes may have an important role in gamete-oviduct interactions and fertility. Moreover, embryos can take up exosomes released from other embryos during the *in vitro* culture as a way of embryo-embryo communication (Saadeldin *et al*., 2014). Ultimately, trophectoderm ovine cells, an established Tr1 cell line from day 15 conceptuses, internalized exosomes collected from uterine fluids (Ruiz-Gonzalez *et al*., 2015). However, the possibility that the early developing embryo takes up exosomes from the oviductal fluid or the OEC internalize embryo-derived exosomes, to the best of our knowledge, has not yet been shown.

Why should we snoop on these conversations? Why does it matter what they say?

By snooping on the private conversation between the oviduct and gametes/embryo a number of genes and proteins participating in these oviductal interactions have been revealed. While the biological nature of this oviductal cross-talk with gametes and embryos is interesting for its own sake, knowing the molecules and mechanisms that pilot these processes

offers great opportunities for the improvement of assisted reproductive technologies (ARTs).

The use of ARTs such as intracytoplasmic sperm injection (ICSI), or *in vitro* fertilization (IVF), bypasses the early maternal interactions in the oviduct**.** Despite all our efforts in improving the procedures or culture media used in these techniques, evidence has shown genomic imprinting disorders (Cox *et al*., 2002; Le Bouc *et al*., 2010; Lazaraviciute *et al*., 2014). Since there is a lack of oviductal interactions in these scenarios, harnessing the molecular clues obtained from snooping on the conversation between the oviduct and gametes/embryos could improve the success of ARTs.

Here a few examples: (i) A solid molecular basis of the maternal mechanisms involved in sperm selection will help to develop advanced selection methods on sperm quality and improve ART outcomes and animal breeding efficiency; (ii) Identify oviductal proteins that enhance sperm survival, will offer great opportunities for the development of long-life semen diluents; (iii) Determining oviductal proteins that support the development of the early embryo will be used in designing new *in vitro* culture media or in reformulating the current ones.

Although the idea of using the identified oviductal proteins seems quite straightforward, in practice, it is not. Some hurdles need to be overcome: the difficulty in the isolation of oviductal proteins; the fact that once the proteins are isolated they may not exert the same effect as *in vivo*; and the fact that gametes and embryos are remarkably resistant towards the uptake of exogenous substances, including drugs, biomolecules, and intracellular markers.

In this regard, the exosomes represent ideal natural nanoshuttles for carrying specific *in vivo* molecules that are not expressed in the *in vitro* cultures. Exosome supplementation will bring a "cocktail" of *in vivo* oviductal proteins, miRNA and lipids to overcome the *in vitro* cultures deficiencies and promote successful pregnancy. Increasing our understanding of the exosome/microvesicle content and function will highlight the great potential for the use of these vesicles as non-invasive biomarkers or as therapeutic assets in infertility and early pregnancy loss.

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Updates on embryo production strategies

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Abstract

The embryo production technologies are used to enhance genetic progress through female and male lineages. Advances in the control of ovarian follicular wave emergence, superstimulation and ovulation with self-appointed treatments have facilitated donor and recipient management. However, these procedures can be influenced by several factors related to the animals and their management. Therefore, researchers continue to investigate the ideal reproductive environments and treatments to maintain the viability of the techniques and field applicability.

Keywords: ET, IVP, OPU, SOV, synchronization.

Introduction

Among reproductive technologies, *in vivo* and *in vitro* embryo productions have been robust tools used to enhance genetic progress through female and male lineages. However, there is an ongoing need to simplify bovine superovulation (SOV) for *in vivo* embryo production, specifically by reducing the number animal handlings, without compromising embryo yield. Advances in the control of ovarian follicular wave emergence and ovulation with self-appointed treatments have facilitated donor and recipient management. However, these procedures can be influenced by several factors related to the animals and their management (Mapletoft *et al*., 2002; Baruselli *et al*., 2006, 2010; Bó *et al.*, 2006; Vasconcelos *et al*., 2006). Therefore, researchers continue to investigate the ideal reproductive environments and treatments to maintain the viability of the techniques and field applicability.

Nowadays, 40.6% of the total embryo production in the world (1,275,874 embryos) are *in vitro* derived embryos (International Embryo Transfer Society - IETS, 2014). The success of *in vitro* embryo production (IVP) is directly related to the number and quality of the cumulus-oocyte complexes (COC) harvested by the ovum pick-up (OPU) procedure. Therefore, several studies have been performed to obtain the expertise related to oocyte quality and consequently, increase the outcomes of OPU-IVP large scale programs.

In this context, the present review aims to discuss some key points relating to genetics, breed, antral follicle populations, manipulation of ovarian follicular growth, frequency of OPU procedures and extrinsic factors (nutrition and heat stress) which are associated with oocyte and embryo quality for *in vivo* and *in vitro* procedures. Lastly, with this awareness of factors related to the efficiency of OPU-IVP, strategies to optimize the technology and its outcome will be discussed.

Strategies for *in vivo* embryo production

Superovulation of Bos taurus *and* Bos indicus *donors without estrus detection*

Traditional SOV protocols have some limitations, including the necessity of numerous animal handling events and detecting estrus to establish the stage of the estrous cycle for initiating superstimulatory treatments and to determine the time of AI. However, recent protocols have been designed to control follicular wave emergence and ovulation, allowing the initiation of superstimulatory treatments and the AI of donors at a self-appointed time (Bó *et al.*, 2006). Protocols for SOV without estrus detection are especially important when working with *Bos indicus* donors and high-yielding dairy *Bos taurus* cows, due to the inherent difficulties with estrus detection with these animals (Lopez, 2005; Baruselli *et al*., 2006).

Thus, three important aspects should be considered when developing SOV protocols: 1) control of ovarian follicular dynamics and follicular wave emergence to initiate gonadotropin treatments; 2) time of ovulation induction and AI in superstimulated donors; and 3) type (FSH or eCG), dosage, and frequency of gonadotropin treatments used for SOV.

Synchronization of follicular wave emergence to initiate gonadotropin treatments

Follicular wave emergence for SOV can be controlled mechanically (follicle ablation; Kohram *et al*., 1998) or pharmacologically (GnRH; Kohram *et al*., 1998), LH, hCG, or estradiol plus progesterone (Bo *et al.*, 1995). In general, the most common treatment to

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electively induce follicular wave emergence involves the use of estradiol and progesterone (P4), especially in *Bos indicus* cattle, because of their prolonged anestrus and poor response to GnRH treatment at random stages of the estrous cycle (Baruselli *et al.*, 2003). The efficacy of estradiol and P4 administration, followed by the initiation of FSH treatment at the expected time of follicular wave emergence (4 days later), has been demonstrated in several studies with *Bos taurus* (reviewed by Bó *et al*., 2006) and *Bos indicus* (Nogueira *et al.*, 2002) cattle. Regardless of the stage of the estrous cycle, estradiol benzoate (EB) treatment at P4 administration (either a norgestomet ear implant or P4-releasing intravaginal device) induces synchronous follicular wave emergence approximately 3 to 4 days after treatment (reviewed by Baruselli *et al*., 2006). Therefore, co-treatment with estradiol and P4 has been considered the most successful hormone therapy to synchronize follicular wave emergence in cattle (Bó *et al.*, 2002).

Time of ovulation induction and AI in superstimulated donors

Although the control of follicular wave emergence allows for self-appointed initiation of gonadotropin treatments for superstimulation, the need to detect estrus to perform AI in superstimulated donors remains an important challenge. Therefore, several studies have been conducted to investigate the pharmacological control of the time of ovulation in superstimulated cattle, thus enabling timed AI (TAI). The interval to ovulation can be controlled by delaying the removal of the progestin/progesterone implant and administration of GnRH/LH at the end of SOV protocol (Bó *et al.*, 2002). Moreover, postponing the LH surge in relation to PGF2α treatment allows the development of more follicles that acquire the capacity of ovulation, thereby resulting in more embryos (Rieger *et al.*, 1990; Vos *et al.*, 1994).

Studies directed toward the development of SOV protocols that allow TAI in various breeds of cattle treated with P4-releasing devices and EB on the first day of the protocol (day 0) have been reported (Baruselli *et al*., 2006; Bó *et al*., 2006). Protocols are named according to the interval from the first PGF2α treatment to the time of P4 source removal (i.e, P-24 and P-36), which occurs before the induction of ovulation to avoid the deleterious effect of high P4 concentration on oocyte quality and sperm transport during the ovulation period (Nogueira *et al*., 2002; Barros *et al*., 2010). No significant differences in the number and quality of transferable embryos have been detected between the P-24 and P-36 treatments (Baruselli *et al.*, 2006). Therefore, both treatments can be used to superovulate *Bos taurus* and *Bos indicus* cattle with TAI.

However, due to differences in the diameter of the dominant follicle at deviation and ovulation in Nelore (smaller) than Holstein (greater) cows (Sartori *et al*., 2001; Gimenes *et al*., 2008), it is understandable that the appropriate time to induce ovulation may differ. Therefore, treatment with GnRH or pLH to induce ovulation for TAI in superstimulated *Bos indicus* and *Bos taurus* donor cows should be done at 12 and 24 h, respectively, after the last FSH treatment (Baruselli *et al*., 2006; Bó *et al*., 2006).

Type (FSH or eCG), dosage, and number of gonadotropin treatments used for superovulation

A series of studies were conducted aiming to reduce the number of animal handlings that were required to induce SOV in *Bos indicus* donors (Baruselli *et al.*, 2008). The use of 3 doses of pFSH resulted in similar embryo production to the traditional 8 dose protocol with pFSH (Martins *et al.*, 2008). Based on the studies reviewed above, it was possible to reduce the number of animal handlings to complete the SOV protocol by reducing the number of FSH treatments in *Bos indicus* donors. However, results may be different in *Bos taurus* cows, which are less sensitive to exogenous gonadotropins (Bó *et al.*, 2006).

Considering the need to develop a simplified superstimulation protocols, studies have focused on alternative methods to maintain FSH release during a prolonged period in *Bos taurus* breeds. An alternative that has been studied for *in vivo* embryo production is the use of a single injection of pFSH in a 2% hyaluronan solution (biodegradable polymer; Tríbulo *et al*., 2011). Vieira *et al*. (2015; Department of Animal Reproduction, USP, São Paulo, SP, Brazil; unpublished data) also reported extended elevated FSH concentrations in Holstein heifers treated with pFSH combined in a 0.5% hyaluronan solution. Previous studies reported a similar number of transferable embryos when a single (in 2% hylauronan; Tríbulo *et al*., 2011) or two (in 0.5 or 1% hyaluronan; Tríbulo *et al*., 2012) IM injections of pFSH was administered compared to the traditional twice-daily IM injections of pFSH.

In order to reduce donor handling, a series of studies were designed to evaluate superstimulatory response after a single intramuscular injection of equine chorionic gonadotropin (eCG) in the P-36 (36 h between the first PGF2α treatment and P4 source removal) protocol with FTAI in Nelore and Holstein donors (reviewed by Baruselli *et al*., 2008). Collectively, treatment with eCG (1,500, 2,000 or 2,500 IU) resulted in a similar number of transferable embryos compared with eight decreasing doses of pFSH. However, a dose of 2,500 IU of eCG resulted in several large anovulatory follicles observed on the day of ova/embryo recovery in Nelore cows. The eCG treatment effectively reduced donor handling, but lower doses of eCG to SOV *Bos indicus* cows are recommended to avoid excessive superstimulatory responses. In *Bos taurus* dairy cows (Holsteins; reviewed by Baruselli *et al*., 2008), treatment with eCG (2,000 or 2,500 IU) resulted in a similar number of transferable embryos compared with eight decreasing doses of pFSH. However, successive treatments with eCG after the third session resulted in

reduced embryo production. Based on these data, we concluded that it was possible to SOV donors with a single dose of eCG but decreasing embryo production is likely to occur with repeated use.

Strategies for *in vitro* embryo production

Manipulation of follicular dynamics

Different reproductive biotechnologies have been used to manipulate follicular dynamic and improve results of OPU-IVP. Ovarian follicular wave dynamics prior to the OPU can be manipulated mechanically (follicle ablation) or pharmacologically (GnRH, LH, hCG, or estradiol/P4). After follicular ablation, a new follicular wave will emerge 1 to 1.5 days later (Adams *et al.*, 1992). Following the E2/P4 treatment, a new follicular wave will emerge 3 to 4 days after treatment (Bó *et al*., 2002; Sá Filho *et al.*, 2013). Martins *et al*. (2012) evaluated the effect of synchronizing follicular wave emergence in Nelore (*Bos indicus*), Brangus (crossbred) and Holstein (*Bos taurus*) cows on the success of OPU programs. Donors were assigned to four groups: control (OPU at a random day of the estrous cycle), D1 (OPU 1 day after follicular wave emergence), D1+bST (OPU 1 day after follicular wave emergence associated to bST administration on day -5) and D1+ eCG (OPU 1 day after follicular wave emergence in association with administration of 400 IU of eCG on day -3). Overall, the eCG treatment resulted in a greater number of viable oocytes in Brangus and Holstein donors. However, the eCG and bST treatments resulted in a greater total number of blastocystsper OPU session only in Holstein donors.

The effect of follicular ablation (aspiration of all >8 mm follicles) or EB+P4 to synchronize follicular wave emergence prior to the OPU was evaluated (Rodriguez *et al.*, 2011). In this study, the effect of eCG or pFSH to superstimulate follicle growth, on the number and quality of COC obtained by OPU in Brangus and Angus donors was evaluated. No significant effects of follicular wave synchronization was detected in the total number of follicles suitable for OPU (14.8 ± 1.2 *vs*. 14.5 ± 1.4), number of retrieved COC (8.3 ± 0.9 *vs*. 7.8 ± 1.0) or number of cultured COC (5.3 ± 0.8 *vs*. 4.8 ± 0.8). However, a greater number of follicles suitable for OPU (18.2 ± 1.1 *vs*. 11.2 ± 1.0) and total number of COC retrieved (9.7 ± 1.0 *vs*. 6.3 ± 0.8) and cultured (6.8 ± 0.8 *vs*. 3.3 ± 0.5) was observed in cows treated with pFSH compared to eCG.

In a recent study, our research group evaluated the effect of superstimulation in lactating and non-lactating Holstein donors submitted to synchronization of follicular wave emergence (Vieira *et al.*, 2014a). Superstimulation with twice daily injections of pFSH over 2 days prior to OPU resulted in greater OPU-IVP efficacy, regardless donor category (Table 1). Regardless of superstimulation treatment, non-lactating Holstein donors had superior IVP outcomes compared to lactating donors (Table 1). A second experiment was performed to evaluate the efficacy of a single IM injection of pFSH combined with 0.5% hyaluronan (MAP-5) in non-lactating Holstein donors submitted to OPU-IVP procedures (Vieira *et al.*, 2015; Department of Animal Reproduction, USP, São Paulo, SP, Brazil; unpublished data). Regardless of superstimulation treatment protocol, greater numbers of blastocysts were produced per OPU session (P = 0.06) in donors receiving pFSH (Control: 2.4 ± 0.5; 200 pFSH: 3.7 ± 0.7; 200 pFSHHA: 4.7 ± 0.7; 300 pFSHHA: 3.1 ± 0.6). Results demonstrate that superstimulation protocols for OPU/IVP programs in Holstein donors will increase embryo yield, and that this can be accomplished with reduced numbers of animal handling (6 to 3) when pFSH is administrated in a 0.5% hyaluronan solution.

Table 1.Summary of oocyte and embryo production(mean ± SE) after OPU-IVP in Control and pFSH-treated donors (lactating and non-lactating Holstein cows).

	Lactating cows		Non-lactating cows		P-value[5]		
	Control	pFSH	Control	pFSH	Treatment	Categ	Treat*Categ
No.	15	15	15	15	.	.	.
Total follicles aspirated	17.6 ± 1.6	18.2 ± 2.1	16.7 ± 1.5	16.3 ± 1.6	0.92	0.52	0.62
Total oocytes retrieved	13.0 ± 1.7	10.7 ± 1.5	10.9 ± 1.6	9.9 ± 1.5	0.10	0.51	0.54
Recovery rate, %[1]	73.9 (195/264)	59.0 (161/273)	65.6 (164/250)	61.1 (149/244)	<0.001	0.89	0.08
COCs cultured	10.0 ± 1.3	8.9 ± 1.3	8.5 ± 1.4	8.3 ± 1.3	0.52	0.58	0.57
COCs culture rate, %[2]	76.9 (150/195)	82.6 (133/161)	78.0 (128/164)	83.9 (125/149)	0.05	0.77	0.88
Cleavage rate, %[3]	65.3 (98/150)	63.2 (84/133)	72.7 (93/128)	72.8 (91/125)	0.81	0.16	0.69
Blastocyst rate, %[4]	10.8 (15/150)	17.3 (23/133)	31.3 (40/168)	52.8 (66/125)	<0.001	0.001	0.16
Embryos produced per OPU	1.0 ± 0.4	1.5 ± 0.5	2.7 ± 0.6	4.4 ± 0.8	0.01	0.003	0.17

[1]No. COCs /no. follicles aspirated; [2]No. COCs cultured /no. total COCs retrieved; [3]No. cleaved zygotes /no. oocytes cultured; [4]No. blastocysts /no. oocytes cultured; [5]Treatment = effect of treatment (Control *vs*. pFSH); Categ = effect of donor lactation status (lactating *vs*. non-lactating); Treat*Categ = interaction between treatment and donor lactation status. Adapted from Vieira *et al*. (2014a).

Factors that influence the efficiency of OPU-IVP programs

In cattle, factors such as genetics or breed, heat stress, nutrition and stage of the estrous cycle can significantly influence the response to different reproductive biotechnologies. For example, in relation to OPU-IVP, it has been reported that IVP is greater in *Bos indicus* breeds than in *Bos taurus* breeds (Pontes *et al.*, 2010; Guerreiro *et al*., 2014).The greater population of antral follicles found in *Bos indicus* cattle would appear to result in a greater number of suitable oocytes for *in vitro* culture (Batista *et al.*, 2014). In this context, *Bos indicus* females are reported to produce a greater number of total and cultured COC and greater blastocyst rates (Gimenes *et al*., 2015; Table 2) than *Bos taurus* females.

Table 2. Effect of genetic groups on oocyte recovery and quality, and developmental competence in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers.

	Genetic group	
	Nelore (n = 9)	Holstein (n = 9)
Number of replicates	6	6
Number of OPU sessions	54	54
Oocyte recovery and quality		
Visualized follicles	41.0 ± 2.1[a]	22.1 ± 1.3[b]
Total oocytes	37.1 ± 2.6[a]	15.4 ± 1.2[b]
Recovery rate (%)	82.3[a]	66.8[b]
Oocytes submitted to IVC	25.6 ± 1.8[a]	9.1 ± 0.9[b]
Developmental competence		
Cleaved structures	21.1 ± 1.6[a]	5.2 ± 0.5[b]
Cleavage rate (%)	82.6[a]	59.9[b]
Blastocysts 7 day after IVF	7.3 ± 0.9[a]	1.1 ± 0.2[b]
Blastocyst rate (%)	28.3[a]	14.1[b]

[a,b]P < 0.05. Adapted from Gimenes *et al*. (2015).

One factor related to the poor IVP yields in *Bos taurus* cattle can be partly attributed to the heat stress (HS; Al-Katanani and Hansen, 2002; Al-Katanani *et al*., 2002; Ferreira *et al*., 2011), mainly in tropical regions. However, previously reports have also shown that HS can exert a deleterious effect on ovarian follicular dynamics and oocyte competence *Bos indicus* cattle as well (Torres-Júnior *et al.*, 2008).

A previous seasonal experiment demonstrated that once the pool of ovarian oocytes is damaged by heat stress, two or three estrous cycles are required (after the end of heat stress) to restore the follicular pool and oocyte quality (Roth *et al.*, 2001). However, the study described above (Torres-Júnior *et al.*, 2008) showed a carry-over effect on blastocyst production up to 105 days after the end of the heat stress (Fig. 1). Therefore, it seems that follicles and oocytes are damaged by heat stress during early stages of folliculogenesis, with a delayed deleterious effect on ovarian function. Nevertheless, *Bos indicus* breeds have been shown to be more resistant to tropical conditions (i.e. elevated temperature and humidity) than breeds that evolved in temperate climates (i.e, *Bos taurus,* as Holstein). Essentially, the adaptation of certain breeds to elevated heat and humidity is related to their ability to thermoregulate their body temperature (Bennett *et al*., 1985; Hammond *et al*., 1996; Gaughan *et al*., 1999).

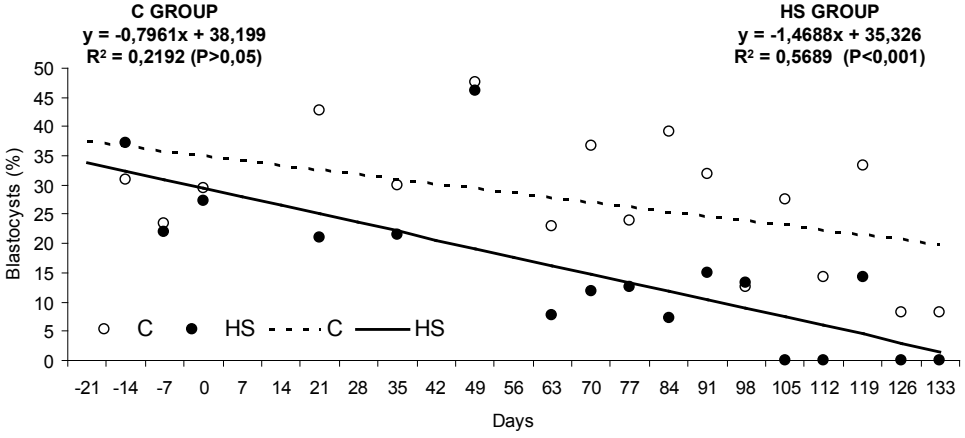

Figure 1. Percentage of blastocysts and regression equation's adjusted lines of oocytes recovered from Gyr (*Bos indicus*) cows exposed to thermoneutral (C) or heat-stress (HS) treatments. Adapted from Torres-Júnior *et al*. (2008).

Heat stress also has a deleterious effect on superovulatory response in Holstein donors. In a recent retrospective analysis, Vieira *et al*. (2014b) reported a negative effect of the warm season in Brasil on the number of *in vivo* produced embryos (2.8 ± 0.3 *vs*. 4.4 ± 0.4; P = 0.03) and percentage of embryos classified as grade I and II (21.4 *vs*. 32.8%, P < 0.0001) in Holstein donors. In addition, Ferreira *et al*. (2011) reported decreased COC numbers in Holstein cows when OPU was performed during the summer months. Yet, when blastocyst rates were evaluated, an interaction between group and season indicated that the effect of season was dependent on animal category. In the summer, blastocyst rate dropped in repeat breeder cows (usually females during late lactation) in comparison to winter, becoming lower than in cows at peak of lactation. However, regardless of season, blastocyst rates were lower in repeat breeder cow than in heifers. Additionally, repeat breeder blastocyst quality was compromised in comparison to heifers and cows at peak lactation during the summer,

A common aspect of commercial OPU-IVP programs is the use of non-lactating or late lactation cows as oocyte donors. In these animals, in addition to the effects of heat stress, diet may also influence IVP. In these animal categories, the negative effects of overfeeding (excessive energy intake) can compromise *in vitro* oocyte developmental competence, especially in over-conditioned (high body condition score) females (Adamiak, 2005). The mechanisms that mediate these negative effects on oocyte competence may be related to endocrine alterations, such as hyperinsulinemia, peripheral resistance of insulin, and increased glucose and IGF-I, which may interfere with glucose transport in embryo cells and increased apoptosis.

The nutritional and metabolic state can interfere with follicular growth patterns, secretion of reproductive hormones, and oocyte quality in cattle (Leroy *et al*., 2008; Ashworth *et al*., 2009; Batista *et al*., 2013; Sales *et al*., 2015). Thus, metabolic imbalances may cause systemic alterations that can compromise the success of reproductive biotechnologies, such as OPU-IVP (Webb *et al*., 2004; Adamiak, 2005). Our research group conducted a study to evaluate the impact of different energy intakes on metabolic profiles and oocyte quality of the non-lactating Gyr (*Bos indicus*) cows submitted to successive OPU sessions (Sales *et al*., 2015). Diets were formulated to achieve maintenance (M) or 1.7% of maintenance (1.7M) for non-lactating cows. We observed that following 60 days of high energy feeding, cows had reduced *in vitro* oocyte competence (Fig. 2). All cows fed high energy diets had greater glucose and insulin concentrations and a greater level of insulin resistance as determined by the glucose tolerance test. Furthermore, cows receiving high energy diets, had a lower abundance of transcripts for GLUT 1, IGF 1R, IGF 2R and HSP70.1 genes in oocytes.

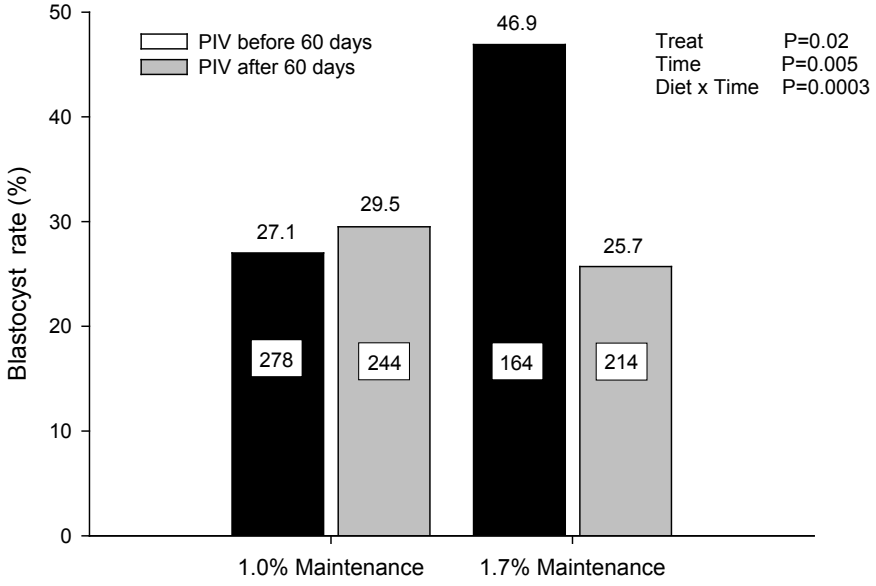

Figure 2. *In vitro* embryo production in non-lactating cows(n = 14) fed diets to meet 100 or 170% of energy of maintenance and submitted to nine OPU session at 14 day intervals. Adapted from Sales *et al*. (2015).

The stage of the estrous cycle at the time of OPU can also influence recovery rates, oocyte quality and *in vitro* embryo production (Hendriksen *et al*., 2000; Merton *et al*., 2003; Vassena *et al*., 2003; Machatkova *et al*., 2004). The number of COC retrieved was greater when the OPU is performed during the early

phase of the follicular wave (Machatkova *et al.*, 2004), probably due to the greater recovery rate after the aspiration of small follicles (4 mm; Seneda *et al*., 2001). However, despite the lower recovery rates, higher *in vitro* competence was observed when oocytes were obtained during early dominance phase of the dominant follicle (Merton *et al*., 2003; Vassena *et al*., 2003; Hendriksen *et al*., 2004). Similarly, stages of the follicular wave affected the numbers of oocytes recovered and *in vitro* competence following OPU in Nelore cows (Melo, 2007). Higher numbers of COC were recovered and *in vitro* embryo production was higher when Nelore cows were subjected to OPU on days 3 and 5 compared to days 7 and 9 after follicular wave emergence.

Based in the protocols described above, our research group designed a trial to evaluate different times relative to follicular wave emergence to perform the OPU-IVP in crossbred (*Bos taurus* x *Bos indicus*) heifers (Gimenes *et al.*, 2007). Follicular wave emergence was synchronized with EB and P4 in all donors and OPU was done 2, 4 and 6 days after follicular wave emergence. Higher blastocyst and hatched blastocysts rates and higher numbers of nuclei in hatched blastocysts were observed when OPU was done on day 2 of the follicular wave. However, no effect of follicular wave status was observed in either Holstein (*Bos taurus*) or Nelore (*Bos indicus*) donors in a subsequent study (Gimenes *et al.*, 2015).

The unexpected effects of day of the follicular wave on OPU-IVP variables may be due to the method of synchronization of follicular wave emergence (pharmacologic synchronization *vs*. follicular ablation). Although our purpose was to provide a more practical method of synchronization of follicular wave emergence, the pharmacologic induction of follicular wave emergence could result in a cumulative follicle population containing follicles undergoing regression together with the new follicle cohort. Therefore, more studies must be conducted to clarify this matter.

Conclusion

The success of *in vivo* and *in vitro* embryo production is closely associated to oocyte and embryo quality. Therefore, factors related to breed, heat stress and nutrition should be considered before applying SOV or OPU-IVP in the field. Adequate control of environmental and nutritional conditions should be one of the requisites to be accomplished before implementing any reproduction biotechnology. On the other hand, strategies established to manipulate follicular wave dynamics (synchronization of the follicular wave emergence and superstimulation) can optimize the efficiency of embryo production techniques. Once these biotechnologies can be efficiently applied on a large scale in the field, significant enhancements in livestock genetic gain can be accomplished bringing productivity and economic return for the activity.

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In vitro and *in vivo* embryo production in cattle superstimulated with FSH for 7 days

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Abstract

Over the past two decades, research efforts have resulted in superstimulation protocols that are user-friendly, but embryo production has increased only marginally. Studies to-date have not adequately answered the question of whether superstimulatory protocols can be used to overcome the follicle wave pattern, increase the number of follicles that enter the wave, or rescue a greater number of small follicles within the wave. Studies which appear to facilitate greater utilization of follicles within the wave are described in this review. The number of large follicles at the time of first AI tended to be greater, and more ovulations and CL occurred with lengthened protocol (7-day) than with the convention 4-day FSH treatment. In addition, there was greater synchrony of ovulations and the mean numbers of total ova/embryos, fertilized ova, transferable or freezable embryos were numerically higher in the 7-day group. When used in an *in vitro* fertilization model, FSH treatment for 7 days resulted in a greater number of follicles for aspiration, a greater proportion of expanded cumulus-oocyte-complexes, and more transferable embryos after *in vitro* culture. Daily ultrasonography revealed a reduction in the number of small (1-2 mm) antral follicles from the beginning to the end of the superstimulatory treatment that was associated with a progressive shift of follicles to the next size category in both 4-day and 7-day groups. The number of follicles $\leq$5 mm decreased during superstimulation suggesting that there was no continuous recruitment of small follicles, and the number of follicles $\geq$1 mm at the end of superstimulation did not differ from the number of follicles $\geq$1 mm at the beginning of superstimulation. However, the total number of follicles $\geq$3 mm at the end of superstimulation, was greater than the number of follicles $\geq$3 mm at the beginning of superstimulation due to growth of the 1-2 mm population into larger size categories during treatment. Results support the hypothesis that both 4-day and 7-day superstimulatory treatment protocols result in rescue of small antral follicles present at the time of wave emergence. However, the lengthened superstimulatory treatment protocol resulted in more follicles acquiring the capacity to ovulate with an increased number of ovulations, and a tendency for increased embryo production without a decrease in oocyte/embryo competence.

Keywords: cattle, follicular development, FSH, oocyte competence, superstimulation protocol.

Introduction

Improvements in our understanding of ovarian function, and gonadotropin preparations has led to important changes in strategies used for superovulation in cattle. Advances in superstimulatory treatment protocols have included the ability to initiate treatments at a self-appointed time (Bó *et al*., 1995; Mapletoft and Bó, 2012), the use of fixed timed AI (Bó *et al*., 2006), and a reduction in the number of FSH treatments (Bó *et al*., 1994; Tríbulo *et al*., 2012; Carvalho *et al*., 2014), all of which have made protocols easier to implement. More recently, it has been revealed that the superovulatory response is related to the number of follicles present at the time of wave emergence and that this number is inherent and repeatable within individuals (Singh *et al*., 2004; Ireland *et al*., 2007).

The number of transferable embryos is defined ultimately by the superovulatory response, fertilization rate and subsequent embryo development. The objective of this manuscript is to review recent thoughts on follicular growth and ovulatory response during superstimulation, and new information on how the duration of the superstimulatory treatment protocol might optimize donor response.

Follicle recruitment and initiation of FSH treatments

During emergence of follicular waves in cattle, a variable number of 3-5 mm follicles appear over a period of 2 to 4 days (Ginther *et al*., 1989; Lucy *et al*., 1992; Adams *et al*., 2008), as a result of a surge in circulating FSH 1 to 2 days earlier (Adams *et al*., 1992). Improvements in the resolution of ultrasound equipment revealed that small antral follicles (1-3 mm) also develop in a wave-like pattern in response to increases in endogenous FSH (Jaiswal *et al*., 2004). The number of 1-3 mm follicles was maximal 1 to 2 days before the previously defined day of wave emergence and was temporally associated with the earliest rise in the pre-wave surge of FSH. In addition, the future dominant follicle was first detected at a diameter of 1 mm approximately 66 h before the previously defined day of wave emergence (based on detection of the future dominant follicle at a diameter of 4 mm), and coincident

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with the beginning of the FSH surge. This discovery of an extension of the wave pattern to include follicles at a smaller developmental stage has important implications for ovarian superstimulation.

The presence of a functional dominant follicle was found to suppress the superovulatory response (Guilbault *et al*., 1991; Bungartz and Niemann, 1994), and it has been shown that superstimulatory treatments must be initiated at the time of wave emergence to optimize response (Nasser *et al*., 1993; Adams, 1994). These findings led to the development of methods to synchronize wave emergence before ovarian superstimulation by treatment with a combination of estradiol and progesterone (Bo *et al*., 1995) or by transvaginal ultrasound-guided follicle ablation (Bergfelt *et al*., 1994). In another study, the superovulatory response and the number of freezable embryos were greater when FSH treatment was initiated 2 days rather than 1 day after ovum pick-up (OPU), a procedure that mimics the effect of follicular ablation (Surjus *et al*., 2014). As all follicles $\geq$3 mm were aspirated during oocyte collection, the additional day may have provided time for smaller follicles in the wave to reach an ovulatory stage during superstimulation treatment.

In contrast, preliminary data from others suggest that superstimulation initiated in the presence of a functional dominant follicle resulted in the emergence of a new follicular wave, and that the presence of a progestin implant for 4.5 days during the period of FSH treatment allowed sufficient additional time for the FSH-induced wave of follicles to reach the ovulatory pool, regardless of the fate of the dominant follicle (Bednar and Pursley, 2000). Similarly, in a review of alternative approaches to setting up donors for superstimulation (Bó *et al*., 2008), preliminary data were presented on the effects of a 6-day superstimulation protocol initiated without regard to follicular wave status. Ovarian response, and the number of ova/embryos and transferable embryos did not differ between the extended protocol without synchronization of wave emergence and a conventional 4-day protocol with synchronization. However, studies to-date have not directly addressed the question of whether superstimulatory protocols can be used to overcome the wave pattern (allow superstimulation regardless of wave status), increase the number of follicles that enter the wave, or rescue small follicles within the wave.

Extended superstimulatory treatment protocols

Based on the notion that exogenous gonadotropins can overcome the wave pattern and result in "subordinate follicle break-through", attempts have been made to increase the superovulatory response by adding eCG treatment prior to initiating FSH treatments. Pre-treatment with eCG 2 days before the conventional FSH treatment protocol resulted in a numerically greater number of ovulations (16.1 ± 1.5 *vs*. 12.4 ± 1.4) and transferable embryos (8.1 ± 1.6 *vs*. 6.5 ± 1.3) in an unselected group of donors (Caccia *et al*., 1999), and a significantly greater number of transferable embryos in donors that were defined as poor responders (3.6 ± 0.6 *vs*. 1.0 ± 0.2; Bó *et al*., 2008).

More recently, we evaluated the superovulatory response and embryo recovery in donors treated with either a 4-day or a 7-day FSH superstimulatory treatment protocol (García Guerra *et al*., 2012). Twenty-four beef cows were blocked by number of follicles $\leq$5 mm at the time of wave emergence and placed into either a 4-day or 7-day FSH protocol utilizing the same total dose of 400 mg FSH (Folltropin-V; Vetoquinol Inc/Bioniche Animal Health; Fig. 1).The mean number of ovulations detected by ultrasonography was greater in the 7-day treatment group (30.9 ± 3.9 *vs*. 18.3 ± 2.9; P = 0.01), consistent with a numerically greater number of follicles $\geq$10 mm just prior to ovulation (27.5 ± 4.1 *vs*. 19.5 ± 2.6; P = 0.11; Table 1). Moreover, ovulations occurred more synchronously in the 7-day group (93% of ovulations occurred 12 to 36 h post-LH as compared to 66% in the 4-day group) suggesting that the superstimulated follicles were more mature and capable of responding to an LH stimulus. Although the total number of ova/embryos, fertilized ova and transferable embryos did not differ statistically, all end-points favoured the 7-day group. In addition, when data from cows with fertilization failure were removed, the number of transferable embryos tended to be higher in the 7-day group (7.6 ± 1.7 *vs*. 4.2 ± 1.5; P = 0.07).

In another study (Dias *et al*., 2013a), a 7-day superstimulation protocol was used to investigate the effects of the length of the follicle growth phase under the influence of progesterone on follicle growth, ovulation and oocyte competence. Beef cows were superstimulated with 25 mg twice-daily of FSH for 4 or 7 days. Again, the superstimultory response (number of large follicles just prior to insemination) was greater (P < 0.05) in the 7-day group, and the numbers of ovulations (15.4 *vs*. 11.6) and embryos (6.7 *vs*. 5.9) were numerically higher in the 7-day group.

The duration of treatment appears to be responsible for the increase in the superstimulatory response rather than the FSH dose. In the two studies referred to above, the number of ovulatory-sized follicles just prior to ovulation was greater following 7 days of superstimulation than 4 days, whether the total dose of FSH was greater (Dias *et al*., 2013a) or the same (García Guerra *et al*., 2012). However, further study is needed to determine the optimal dose of FSH when an extended superstimulatory treatment is used.

Table 1. Superovulatory response (mean ± SEM) and ova/embryo recovery in beef cows given a conventional 4-day *vs*. a 7-day superstimulatory treatment protocol.

End point	Treatment group		P-value
	4-day	7-day	
Number of cows	12	12	
Corpora lutea	20.8 ± 2.2	27.2 ± 2.1	0.04
Total ova/embryos recovered	11.3 ± 2.0	13.8 ± 2.3	0.44
Fertilized ova	5.6 ± 1.5	8.0 ± 2.0	0.24
Percentage of fertilized ova	59.0 ± 10.7	54.2 ± 9.5	0.58
Degenerate embryos	1.4 ± 0.7	1.7 ± 0.6	0.73
Transferable embryos (Grades 1-3)	4.2 ± 1.5	6.3 ± 1.6	0.25
Percentage of transferable embryos	44.4 ± 11.8	44.1 ± 8.4	0.69

Adapted from García Guerra *et al*., 2012.

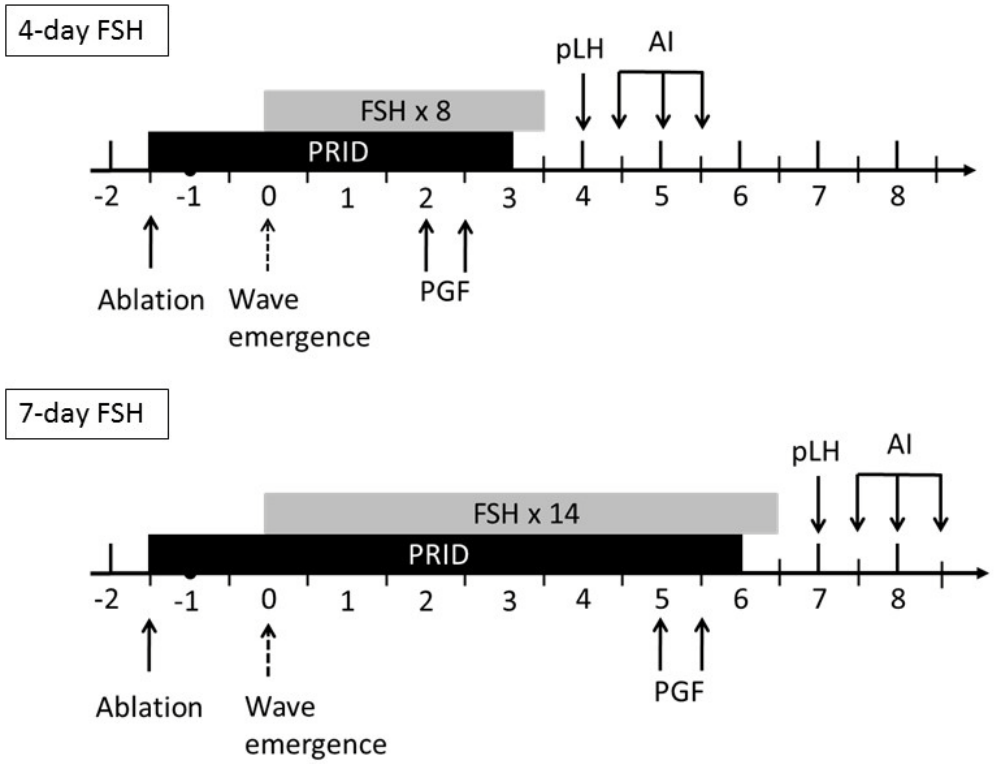

Figure 1. Experimental design used to compare the superovulatory response between a conventional 4-day *vs*. a 7-day superstimulatory protocol in cattle. From García Guerra *et al*. (2012).

Follicle development during superstimulatory treatments

An earlier study provided rationale for a hypothesis that superstimulatory treatment does not recruit more follicles into the wave, but rather permits more small follicles of a wave to attain medium and large diameters (Adams *et al*., 1993). This hypothesis was tested in a recent study designed to compare the effects of a conventional (4-day) *vs*. a lengthened (7-day) superstimulation protocol on follicle dynamics (Garcia Guerra *et al*., 2015). The number of follicles ≥1 mm at the end of superstimulation did not differ from the number of follicles ≥1 mm at the beginning of superstimulation, but the number of small (1-5 mm) antral follicles decreased dramatically during superstimulation. In addition, there was a negative correlation between the number of follicles ≤5 mm and those >5 mm. There was also a greater number of follicles ≥3 mm at the end of superstimulatory treatment

than at the start which was attributed to the rescue of the 1-2 mm population and their growth into larger categories during treatment. These results indicate that superstimulatory treatment rescued small antral follicles within the wave from atresia rather than recruiting more follicles into the wave (Fig. 2).

Although small follicles in the wave appeared to have been rescued in both groups, significant differences were detected in the developmental pattern of the growing cohort (Fig. 2). When the 7-day and the 4-day treatment protocols were compared, the decrease in the number of small follicles and the increase in the number of large follicles tended to occur at a relatively lower accumulated dose of FSH, and the mean growth rate of follicles between initiation of treatment and ovulation was lower (1.3 ± 0.1 *vs*. 1.9 ± 0.1 mm/day) in the 7-day group (Garcia Guerra *et al*., 2015). Because follicles in 7-day protocol grew at a slower rate, and appeared to exist at an ovulatory size for a longer period of time, they may have had more time to acquire the capacity to ovulate, as indicated by the greater number and synchrony of ovulations than in the 4-day protocol (Garcia Guerra *et al*., 2012).

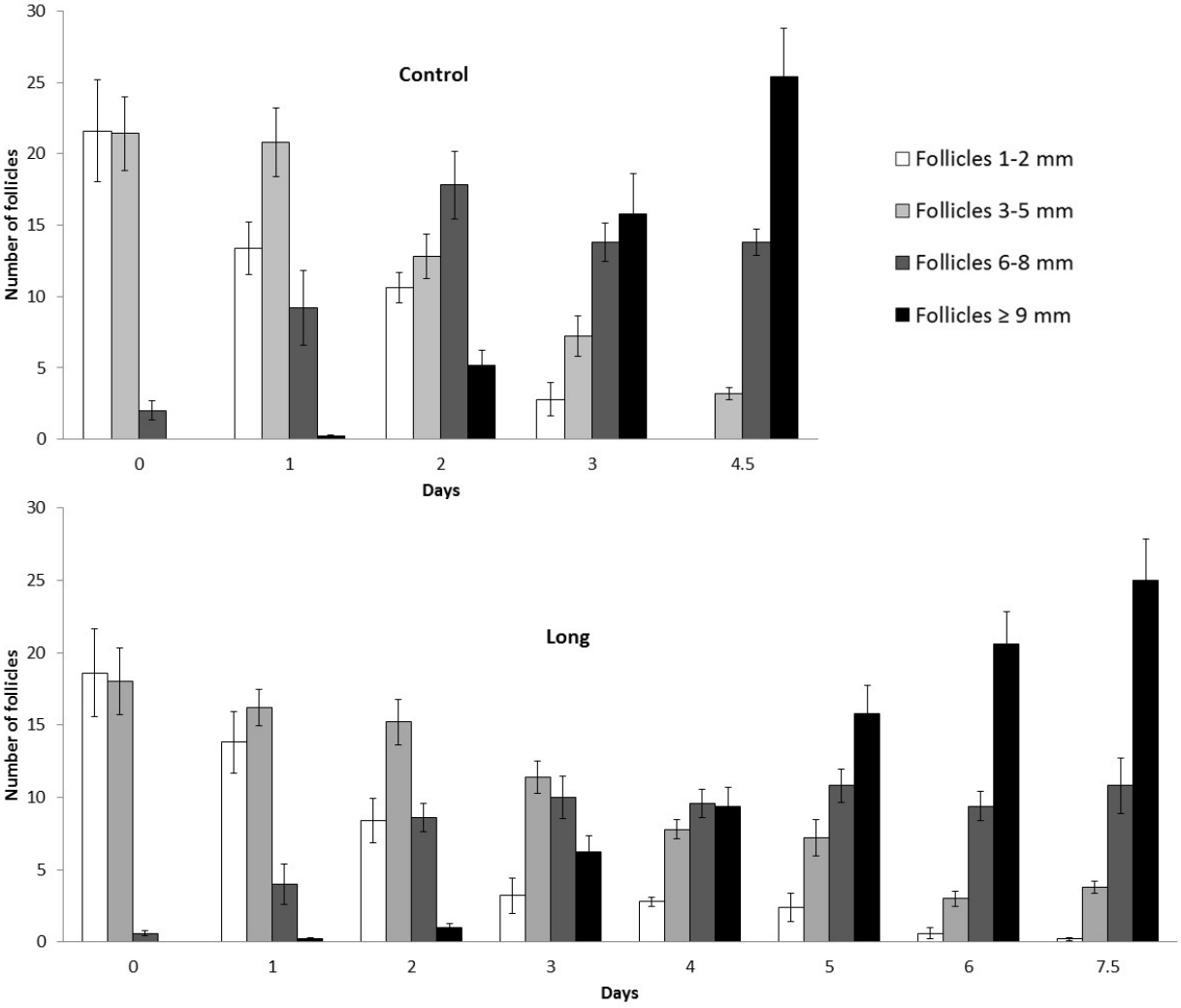

Figure 2. Mean (± SEM) number of follicles in different size categories in cows during a conventional 4-day *vs*. a 7-day superstimulatory protocol in cattle. From García Guerra *et al*. (2015).

Micro-array analysis of large antral follicles

As overcoming follicle selection is a key factor for ovarian superstimulation, exogenous FSH is used to prevent regression of subordinate follicles so that they assume qualities of a dominant follicle. In a recent study of follicles undergoing a 4-day superstimulation protocol, gene expression in granulosa cells was altered compared to a single naturally occurring dominant follicle (Dias *et al*., 2013c, 2014). Expression of growth-related genes (even though LH had been administered), similar to the pre-LH stage of follicle growth, and those involved in oxidative stress response were up-regulated in granulosa cells of follicles

undergoing a 4-day FSH superstimulation protocol compared to a dominant follicle of an unstimulated follicular wave. Genes related to a disturbance in angiogenesis were also up-regulated in superstimulated follicles. We speculate that gene expression during a 7-day superstimulation protocol may be more similar to the naturally occurring dominant follicle.

The acquisition of LH receptors is commonly used as a marker for dominance and a prerequisite for the establishment of ovulatory capacity (Sartori *et al*., 2001; Barros *et al*., 2012). Recently, the evaluation of the expression of LHR mRNA in granulosa cells of superstimulated and unstimulated follicles 12 h after progesterone device removal revealed that expression of LHR was decreased following superstimulation (Lucacin *et al*., 2013). As a result, following the conventional 4-day FSH superstimulation protocol, follicles lagged in maturational development and response to LH compared to a naturally occurring single dominant follicle (Dias *et al*., 2013c, 2014). These findings further support the concept that following a 4-day superstimulation treatment some follicles do not have sufficient time to mature, up-regulate expression of appropriate genes, and acquire the capacity to ovulate.

The use of extended superstimulation protocols for OPU

Most protocols used to stimulate follicle growth prior to OPU are shortened superstimulation protocols with *in vitro* maturation following oocyte collection (Blondin *et al*., 2002). Although we recognize that it is unlikely to be practical in a commercial setting, we have examined the use of an extended superstimulation treatment protocol on *in vitro* embryo production. *Bos taurus* beef heifers were superstimulated using a 4-day or a 7-day FSH protocol and cumulus oocyte complexes (COC) were collected 24 h after LH treatment (Dias *et al*., 2013b). Compared to the traditional 4-day FSH protocol, the 7-day protocol resulted in a significantly greater number of follicles $\geq$9 mm at the time of COC collection (25.4 ± 5.3 *vs*. 10.6 ± 2.3), a tendency for a greater number of COC collected (17.8 ± 3.7 *vs*. 10.5 ± 2.4), and 2.5 times more transferable embryos at the end of *in vitro* embryo culture (9 days after IVF). Interestingly, >90% of the COC collected in the 7-day group were expanded, while in the 4-day group only 74% of the COC were expanded (ns). In another study, the 7-day protocol resulted in the collection of a greater proportion of mature oocytes (59 *vs*. 22%) compared to the 4-day protocol (Dadarwal *et al*., 2014). Although the developmental potential of oocytes was similar between 4-day and 7-day protocols, embryo production was increased in the 7-day group because of a greater number of larger follicles from which COC could be aspirated.

Conclusions

An accumulating body of research suggests that the addition of 2 to 3 days to a traditional 4-day superstimulatory treatment protocol allows time for even the smallest follicles of a wave to be rescued from atresia and incorporated into the cohort that attains ovulatory capability. Conversely, the conventional 4-day superstimulatory treatment protocol results in an accelerated growth rate of follicles that may acquire the capacity to ovulate, but do so less synchronously and may produce oocytes of sub-optimal competence. The extended superstimulatory treatment protocol appears to result in a greater number of transferable embryos both *in vivo* and *in vitro*, because of a greater number of mature follicles. Based on these studies, we conclude that extending the period of follicular development during superstimulation (from 6 to 9 days between wave emergence and ovulation) increases follicular maturation, and the number and synchrony of ovulations without compromising ova/embryo competence, and in turn, the potential for more transferable embryos.

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Genomic selection of *in vitro* produced and somatic cell nuclear transfer embryos for rapid genetic improvement in cattle production

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Abstract

This paper provides basic concepts of genomic selection (GS) methods in beef and dairy cattle production in combination with assisted reproductive technologies (ART) such as ovum-pick up and *in vitro* production (OPU-IVP). We first introduce genomic tools and discuss main methods of GS as practiced to-date. The general benefit from GS is that it enables selecting animals accurately early in life using genomic predictions particularly those phenotypes that are very difficult or expensive to measure. While it is known that GS increases genetic gain and profit in conventional cattle breeding, GS is much more desirable when combined with OPU-IVP in cattle production. The expected benefits of GS-OPU-IVP far exceed the benefits achieved by either GS or OPU-IVP alone mainly due to tremendous reduction in generation interval. The genetic improvement will increase even further, if genetic merit of donor cows and bulls used in OPU-IVP for key economic traits are maximal. The paper also highlights some challenges particularly with regard to embryo biopsies and quantity and quality of embryo DNA for whole genome genotyping and ways to overcome difficulties. We briefly discuss the somatic cell nuclear transfer (SCNT) technique in the context of applying GS on fibroblast cell lines from fetuses obtained from OPU-IVP techniques and provide our perspectives on how it might pave way for even more rapid cattle improvement. Main conclusion is that employing genomic selection in ARTs such as OPU-IVP of embryos coupled with embryo sexing and SCNT will lead to rapid dissemination of high genetic merit animals on a scale never been seen before. Finally, the paper outlines current research activities on combined genomic selection and advanced reproductive technologies in the GIFT project consortium (www.gift.ku.dk).

Keywords: cattle, embryo transfer, genomic selection, OPU-IVP, somatic cell nuclear transfer.

Introduction

Rapid population growth will increase the demand for food as well as other animal products, particularly in emerging economic giants like Brazil and India. Moreover, the urbanization has considerable impact on patterns of food consumption in general and on demand for livestock products in particular. Cattle (dairy and beef) production in most countries in North America and Europe has well established infrastructure and organizational structures to improve economically important animal traits for decades. This has led to substantial increase in both (the efficiency of) meat and milk production from cattle as well as the ability to attain self-sufficiency, but more importantly to a significant source of national income from export and other industries. In sharp contrast, there are several bottle necks in establishing infrastructures and organizational structures for performance data recording in farms and in central test stations/feedlots for calculation of estimated genetic merit (EBVs: estimated breeding values) and applying assisted reproductive technologies (ART) such as *in vitro* production (IVP) of embryos and Embryo Transfer (ET). In addition, both productivity and efficiency of production in developing and/or tropical countries is very low due to environmental stressors and challenges (O'Neill *et al*., 2010).

However, molecular breeding techniques such as combined genomic selection (GS) and modern ARTs such as ovum-pick up and *in vitro* production (OPU-IVP) of embryos provide a rapid and sustainable avenue for genetic improvement of both efficiency and productivity. This is to an extent that it can bypass some of the expensive data recording, progeny or performance testing and conventional genetic evaluations of large number of animals on a routine basis.

The global bovine embryo market reached 1,275,874 embryos during 2013 (International Embryo Transfer Society - IETS, 2014). Importantly, from 2000

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to 2013 the IVP of embryos went from 17.4 to 40.6% (517,587 produced embryos) of representativeness compared to the *in vivo* technology (IETS, 2014). Moreover, this global increase was related to the remarkable improvement in the Brazilian IVP market, responsible for 70.8% of the IVP of embryos (IETS, 2014). Brazil expanded over seven times the bovine IVP from 2001 (50,000 embryos) to 2013 (366,517 embryos). Considering the *in vitro* embryos produced in Brazil, in 2013, 45.7% (167,452 embryos) were obtained from dairy donors (88.6% of *Bos taurus* females) and 54.3% (199,065 embryos) from beef cattle (86.8% of *Bos indicus* females; Viana *et al*., 2015; University of São Paulo, Brazil; unpublished data). Therefore, this IVP index reflects the Brazilian potential market in *Bos indicus* and *Bos taurus* donors submitted to the OPU and IVP programs in large scale.

In addition to large scale increase in embryo production, it is now also theoretically possible to combine GS and OPU-IVP with somatic cell nuclear transfer (SCNT), taking the cattle production well beyond its current potential. The combined GS, OPU-IVP and SCNT, if applied widely, has a tremendous potential for the entire world cattle production including developed countries.

This paper provides basic concepts of using genomic selection (GS) methods applied to OPU-IVP cattle production. The paper also highlights the challenges as well as the expected benefits of genomic selection applied to bovine IVP. We briefly discuss the SCNT technique in the context of applying GS on fibroblast cell lines from fetuses obtained from OPU-IVP techniques and provide our perspectives on how it might pave way for rapid cattle improvement on a scale that has never been seen before. Finally, the Consortium on Genetic Improvement of Fertilization Traits (GIFT) in Brazilian and Danish Cattle (www.gift.ku.dk) and their main activities are mentioned.

Genomicsin cattle production and reproduction

Known genes and genetic markers influencing animal traits allow breeders to make improvements using gene assisted selection and marker-assisted selection (MAS; Kadarmideen *et al*., 2006; Kadarmideen and Reverter, 2007). However, the implementation of MAS programs has rarely been successful for several reasons (Goddard and Hayes, 2009). There are some exceptions to this case, where known genes with known functional impact on the reproduction or fertility are used. For instance, in case of bovine IVP, the oocyte quality and quantity is important. The genes that are predictive of good quality oocyte include FSHR (Izadyar *et al*., 1998), EGFR (Conti *et al*., 2006), AREG (Nautiyal *et al*., 2010; Peluffo *et al*., 2012), PR (Aparicio *et al*., 2011), COX2 (Takahashi *et al.*, 2006), GDF9 and BMP15 (Hussein *et al*., 2006; Gilchrist *et al*., 2008), H2A (Pasque *et al*., 2011), PDE3 (Richard *et al*., 2001) and OOSP1 (Tremblay *et al*., 2006). There are genes that are predictive of good quality blastocysts which include ACSL2 and HAND1 (Arnold *et al*., 2006), G6PD, GPX1, OCT4, PLAC8, SOD2 (Cebrian-Serrano *et al*., 2013), GLUT1, GLUT3, KRT8, PGK1 (Machado *et al*., 2012), GATA6, SOX2 (Ozawa *et al*., 2012), IL1-B (Paula-Lopes *et al*., 1998), LIF, LR-B (Rizos *et al*., 2003).

The Bovine Genome Sequencing and Analysis Consortium initially sequenced and assembled *Bos taurus* cattle genome with approximately 7-fold coverage - this initial assembly reported around 22,000 genes and 14,345 orthologs shared among seven mammalian species (Elsik *et al*., 2009). The benefits of genome sequencing efforts are that it has led to detection of tens of thousands of abundant markers called single nucleotide polymorphisms (SNPs). These abundant SNP markers in several thousands to a million, in the form of genotyping arrays or SNPchips, can be used in whole genomic selection rather than in MAS that uses only a few hundreds genetic markers.

Genomic selection (GS) and its benefits in conventional breeding

Genomic selection relies on 'whole genomic prediction' of breeding values and was coined in the landmark paper by Meuwissen *et al*. (2001). GS methods based on best linear unbiased prediction (BLUP) models enable us to predict the performance of animals given their genotypes at SNPs across the entire genome. These SNP effects are estimated from a large reference population with both genotypes (from SNPchip) and phenotypes of interest. Estimated SNP effects are then used to determine the merit of other genotyped animals that are not yet phenotyped. Common GS methods are Genomic best linear unbiased prediction (GBLUP; Goddard *et al*., 2011), Single-step BLUP (ssBLUP) method (Aguilar *et al*., 2010), and several Bayesian approaches (BayesA, BayesB and BayesCπ; Meuwissen *et al*., 2001; Habier *et al*., 2011).

Genomic prediction models vary based on several assumptions regarding the variance of traits of interest. GBLUP is a prediction method that assumes that all markers contribute to the additive genomic variance. This method is similar to the traditional BLUP method applied for in animal breeding for over 25 years, except that a genomic relationship matrix replaces the numerator relationship matrix computed from the pedigree information. Another method called Random regression BLUP (Meuwissen *et al*. (2001) assumes SNP effects are randomly distributed, and is considered equivalent to GBLUP (Goddard *et al*., 2011). ssBLUP jointly analyzes phenotypes and genotypes of all animals in one step (Aguilar *et al*., 2010). Inclusion of all animals (with and without genotypes) results in the better correction of genomic preselection effects; and consequently provides more accurate estimation of

GEBVs. Several Bayesian approaches have been used for genomic prediction, and these methods assume a prior knowledge about distribution of SNP effects influencing a trait. BayesA assumes that all SNPs have an effect, but each SNP has a different variance that is assumed to be equivalent to a scaled inverse-χ2 prior. The BayesB and Bayes$C\pi$ assume that each SNP has either an effect of zero or non-zero with probabilities π and 1-π, respectively. Genomic prediction accuracy gets better as the trait heritability and the reference population used for calculating GEBVs increases. Overall, genomic prediction methods are improving, especially with advent of whole genome sequence data from next generation sequencing. At present, the difference between performances of different methods is marginal for most traits because they are controlled by many QTLs with small effect sizes. Genomic BLUP and its single step extension that includes non-genotyped animals (ssBLUP), remain the most commonly used methods. A new Systems Genomic BLUP or *sg*BLUP method has been introduced by Kadarmideen (2014) that accounts for biological or functional importance of SNPs in a similar framework as GBLUP.

GS has dramatically changed traditional progeny testing schemes in cattle and other species. This is because GS requires only a smaller proportion of animals to be measured for their performance (production or reproduction ability) and genotyped using SNPchip. It then predicts the performance of large proportion of animals that were not measured for performance but only genotyped. Genetic gain is increased by GS by increasing intensity of selection, accuracy of Genomic EBVs (GEBVs) and genetic variance and by reducing generation interval (Kadarmideen, 2014). Two major advantages of genomic selection compared with traditional selection based on pedigree and phenotypic data alone are (i) it can select animals accurately early in life (even at the embryonic stage) using their GEBVs, (ii) it can also predict phenotypes that are very difficult or expensive to measure, including but not limited to fertility, meat quality, disease resistance, methane emissions, and feed conversion (Hayes *et al*., 2013). In dairy cattle, for example, GS can reduce the generation interval by at least two years as we can pre-select the young bulls to be either progeny tested for production or used directly in the breeding programmes without progeny testing. It is stated that increase in genetic gain or income is 60 to 120% compared to traditional methods of progeny testing (Schaeffer, 2006; Pryce and Daetwyler, 2012), mostly achieved via dramatic reduction in costs of rearing large number of animals and selecting only a few as breeders. A recent study in Brazil (Neves *et al*., 2014) involved assessment of genomic predictive ability for 13 different weight and carcass traits, gestation length, scrotal circumference and two selection indices using 685 Nellore bulls with the Illumina Bovine HD chip SNP data (320,238 SNPs). Their results showed that accuracies of genomic predictions ranged from 0.17 (navel at weaning) to 0.74 (finishing precocity). Across traits, Bayesian regression models (Bayes C and BLASSO) were more accurate than GBLUP. The average empirical accuracies were 0.39 (GBLUP0), 0.40 (GBLUP20) and 0.44 (Bayes C and BLASSO). This study underlined and demonstrated that genomic selection can be practiced in Brazilian Nelore cattle and with this range of accuracy of selection, one can expect similar efficiency and genetic improvement as in Dairy catte and other livestock species.

Genomic selection in bovine IVP of embryos

Merging GSwith IVP production technologies can take the potential genetic improvement well beyond what can be achieved by individual methods alone (either by GS or by IVP). First of all, most genetic studies so far indicated that there is a heritable variation in donor cow's ability to produce good quantity and quality of oocytes (Merton *et al*., 2009) and recipient cows ability to maintain pregnancy and deliver IVP-calves (Spell *et al*., 2001; König *et al*., 2007). The genetic variation and heritability are very important criteria because if no genetic variation or heritability exists for a trait means, there will be no possibility for GS. Fertility in general, is a low heritable trait in both dairy and beef cattle. For instance, (Kadarmideen *et al*., 2000, 2003) reported heritability estimates ranging from 0.05 to 0.16 for traits such as non-return rates or conception rates, days to heat and first insemination, number of inseminations per conception, service period and calving interval in Holstein dairy cattle.

Age at puberty has a major effect on the productive, reproductive, and economic efficiency of female cattle (Monteiro *et al*., 2013). Eler *et al*. (2002) estimated the heritability of 0.57 ± 0.01 for the conception rate of young heifers during exposure to bulls in breeding season; this rather high heritability indicates that genetic selection could be useful to select heifers with a greater probability of precocious fertility. Additionally, studies performed in South America reported a high heritability of age at puberty in Zebu breeds (Nogueira, 2004). Therefore, heifers genetically selected for age at puberty, at first conception, and consequently at first calving should improve reproductive efficiency in cattle herds. These fertility traits would respond to GS due to existence of genetic variation between animals.

With regards to genetic basis of attributes or traits that are important for IVP, Merton *et al*. (2009) analyzed CRV (formally Holland Genetics) data from the OPU-IVP program from January 1995 to March 2006 and reported a heritability of 0.25 for number of cumulus-oocyte complexes, 0.09 for quality of cumulus-oocyte complexes, 0.19 for number and proportion of cleaved embryos at day 4, and 0.21 for number and proportion of total and transferable embryos at day 7 of

culture. These heritability estimates are on par with some of the meat and milk production traits that respond very well to GS.

As described above, the largest increase in genetic gain can be achieved by shortening the generation interval. In the simplest case of application of GS in IVP, an unborn animal's genetic merit is predicted at the embryo stage prior to implantation into recipient cows. The genetic gain is therefore improved rapidly by substantial reduction in generation interval because selection is made on an animal that was never born (Fisher *et al*., 2012; Ponsart *et al*., 2014). Furthermore, IVP embryos will be in large quantities compared to live born animals, therefore only a few animals are selected from large pool of animals (in their embryo stage) based on genetic merit (GEBVs), and rest of the embryos are discarded - this increases the selection intensity rapidly. Both reduction in generation interval and increased intensity of selection will lead to rapid genetic improvement. The large scale application of Genomic Screens of preimplantation Embryos (GSE) depends on cost-benefits of GSE to commercial producers. In fact, the use of sexed semen in IVP and combining this with GSE will transform the cattle industry.

However, GSE before embryo transfer would still be beneficial economically due to costs involved in embryo transfer (ET) of large number of embryos into recipient animals where pregnancy rates differ between uses of cows versus heifers. Further, the cost of raising ET calves of unknown genetic merit with later culling of inferior calves would also result in large logistical costs. For these reasons, GSE and selecting embryos before transfer would maximize the profit for the farmers by only transferring a "reasonable" number of embryos and raising only animals of "reasonable" genetic merit for meat or milk production. In fact, GSE will be very necessary for breeding companies to reduce costs by limiting number of ET and maintenance of unwanted calves.

The whole IVP operations can be further fine-tuned if both donor cows as well as the semen of sires used for the procedure have been genetically evaluated for a number of economically important traits (use only donor cows and sires with high GEBVs in IVP). This does not appear to be an issue for large commercial cattle breeding companies, because all animals in the breeding program go through GS and hence GEBVs should be available. However, the problem comes when IVP companies are not integrated within breeding companies, where IVP companies do not have access to GEBV information on donor cows and semen used in fertilization.

The entire workflow (depicted in Fig. 1) shows that even before GSE, one can only use the high genetic merit donor cows for OPU and use only the high genetic merit bull semen (sexed or unsexed) in fertilization; thus an IVP embryos from these parents are already high genetic merit. However, not all the full-sib embryos from the same parents will have the same genetic merit due to Mendelian sampling variance. Figure 1 illustrates that DNA can be extracted, embryos genotyped and subject to GSE prior to implantation. These assisted reproductive technologies (ART) combined with GS are expected to have dramatic impact in developing countries where traditional animal breeding, improving pregnancy rates via AI and GS is difficult to achieve or implement due to costs and infrastructural constraints. For instance, best bulls and donor cows could be identified within the large private farms or semi-private or private stud breeders or government-owned progeny testing farms. Typically IVP companies, with the help of genetic evaluation labs, can produce and deliver embryos of high genetic merit directly to places where ET takes place. The ET is usually carried out at veterinary dispensaries or hospitals and government AI centers or by technicians employed within large farms. If IVP and GS can be achieved successfully within and across several villages or townships, co-ordinated by regional centers, it will lead to overall genetic improvement rapidly and contribute to food security.

Challenges of GS in bovine IVP of embryos

There are certain technical limitations as to how widely GSE could be practical. There are critical issues in performing embryo biopsies and obtaining sufficient DNA quality and quantity for GS (Ponsart *et al*., 2014). While embryo biopsies for DNA extraction and amplification for genotyping is needed for GS, it has many technical limitations such as reduced genome coverage, allele drop-out at heterozygous loci which leads to lower SNP call rates relative to the threshold standards needed for genomic enhanced genetic analysis, missing genotypes, amplification of artifacts, or allele drop-in (Lauri *et al*., 2013; Kasinathan *et al*., 2015).

In New Zealand, Fisher *et al*. (2012) conducted a genotyping experiment using one- and three-cell biopsies from bovine morulae and using biopsy of trophectoderm from transferable quality blastocyst-stage embryos. The authors concluded that greater numbers of embryonic cells provided in the sample resulted in greater average call rate and lower replication error. The call rate for 30-40 cell embryo samples approached the 99% rates typically achieved for parental DNA obtained from blood. This provides an encouraging result.

Ramos-Ibeas *et al*. (2014) established an *in vitro* culture system to support the growth of bovine trophoblastic cells from an embryo biopsy using different cell sources of conditioned media, eliminating the risk of contamination with feeder cells. They claim that *in vitro* culture system facilitated the establishment of trophoblastic cell lines, which can be expanded (cultured) for more than 2 year and can be useful to studies in relation to placentation processes. In the context of large scale genotyping for GS, this approach

could also be employed to produce a relatively large amount of good quality genomic DNA for bovine embryo genotyping and epigenotyping.

Overall, GSE on OPU-IVP embryos is ongoing already in some private cattle breeding companies in major industrialized cattle producing countries but its widespread application is not yet optimal.

Somatic cell nuclear transfer (SCNT) in genomic selection

Recently, Kasinathan *et al*. (2015) proposed further reduction in generation interval and production of high genetic merit calves by combining advanced reproductive processes such as OPU- *in vitro* fertilzation (IVF) and GS on embryos with somatic cell nuclear transfer (SCNT). Collection of day 21-23 early stage embryos after ET from recipient cows and the establishment of cell lines from these embryos allowed rapid determination of enhanced genetic merit for a large number of candidate embryos. Kasinathan *et al*. (2015) show that fibroblast cell lines established from early stage embryos and subsequent GS on cell lines supported the production of high genetic merit calves by SCNT with efficiency comparable to IVP embryos. This method reduces the generation interval by approximately 7 months and offers the chance to produce multiple animals at the same or later time from banked, frozen fibroblast cell lines. They claimed that this approach is scalable and can lead to considerable savings for breeders by achieving substantial reduction in generation interval and selectively producing animals with the desired genetics within a timeframe of approximately one year.

Implementation

We proposed that overall there are 4 stages where GS can be applied in the entire production chain: First in donor cows, second in the bulls, third in pre-implantation embryos and fourth at the level of fibroblast cell lines. The difference between third and fourth stage is minimal because embryo or fetus genetic make-up are the same, except that a fetus could go through epigenetic and other programming events in the uterus. Hence, due to costs and practical limitations of obtaining adequate DNA for genotyping, third stage GS can be skipped if SCNT will be performed. If SCNT will not be performed, there will be only first three GS stages. At the minimal GS on pre-implantation embryos are highly recommended to improve the average genetic merit of all animals produced via OPU-IVP and reduce costs for the companies and farmers by minimizing unwanted ETs and raising calves with poor genetic potential. An overview of GS of embryos, OPU-IVP, embryos sexing and SCNT for rapid dissemination of high genetic merit animals in cattle production was given in Fig. 1.

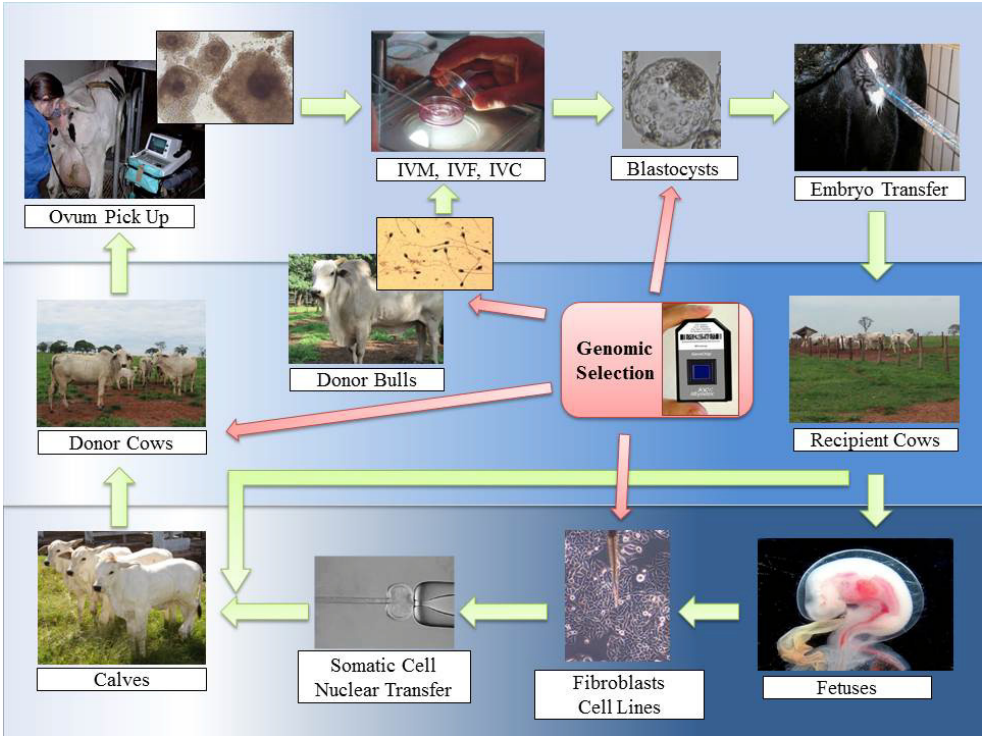

Figure 1. Work flow depicting a cycle of Genomic Selection on donor cows used in OPU-IVP, on donor bulls' semen used in IVF (either sexed or unsexed) and on pre-implantation embryos in rapid production of high genetic merit calves.Alternatively genomic selection is conducted on fibroblast cell lines from fetuses from recipient cows and used in SCNT for rapid production of calves of high genetic merit.

Danish-Brazilian bilateral GIFT project consortium

Much of the reviews provided here in this paper are actually taking place in the Danish-Brazilian bilateral GIFT project (www.gift.ku.dk). Here, we aim to deliver genomic estimating breeding values (GEBVs) and heritabilities (h^2) for key OPU-IVP traits and heifer pregnancy rates and correlations with other traits by conventional genetic evaluation tools. OPU-IVP traits include Oocyte Number (ON), Oocyte Quality (OQ), pregnancy rates in recipient cows as well as normal calving rates. We conduct Genome Wide Association Study (GWAS) to pinpoint genes and genetic variants (SNPs) influencing key IVP traits of cows and heifer pregnancy rates. We hope to deliver necessary information to develop a low density SNPchip which can improve pregnancy rates from 40 to 60% and follicles per ovary from score 1 to 5 of both ovaries. With 600 animals as a base reference population, we plan to conduct whole genomic prediction and genomic selection methods for OPU-IVP traits and with over 2000 Nellore cattle, genomic selection for heifer pregnancy is ongoing.

Legislation, ethics and costs of bovine ART

In Brazil, OPU-IVF is practiced widely and there are no legal barriers. The challenge indeed is in improving the pasture production and quality, feedlot production, Fixed Time Artificial Insemination (FTAI), IVF, ET, Fixed Time Embryo Transfer of IVF embryos, calculation and the use of genetic merit (estimated breeding values) of the cows, heifers and AI bulls in cattle breeding as well as better infrastructure and administration. This will have an important impact in the quality and amount of the Brazilian beef production. More or less, the situation is similar in most developing countries. In Denmark, a number of issues are related to the use of this range of ARTs in cattle breeding and production. Among the ARTs discussed, today's legislation in Denmark only bans the use of SCNT. However, the issue is continuously discussed both in Denmark and in the EU, both in relation to this technology itself, its influence on animal welfare and the possible effect on the resulting food products. How any future modifications will be is not known at present. The ethical discussion is related to both the general view on the techniques, their influence on the animals and the consumer's attitudes on the resulting food products. There is a generally negative view on the increasing use of ARTs in cattle breeding and production, where some of the reasons are related to concerns for the animal welfare. Examples are the repeated use of needles for anesthesia and for oocyte collection in OPU as well as the potential risk for both the recipient and the calf using IVP related to the large offspring syndrome (LOS). In particular issues related to LOS have been resolved to a high degree with the improved serum-free media for embryo culture. On the positive side are the potential achievements from a more powerful genetic selection when the breeding goals are for example focused on less mastitis, stronger legs, less digestive disease etc.

An open and public discussion about these issues is important, and in Denmark the governmental advisory Animal Ethical Council is a key player. Such discussion must have input also from those who actually working with these technologies, and such information can come from anywhere in the world. Therefore, the on-going GIFT research project can be a strong source of information, considering the huge experience found in Brazil on practical use of all these ARTs.

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Oocyte developmental competence and embryo quality: distinction and new perspectives

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Abstract

In vitro embryo production is the cornerstone of infertility treatment in human and is increasingly used in cattle to propagate high genetic merit animals. To increase its efficiency, many different approaches have been tested all of which stem from the concepts of oocyte quality and developmental competence. Presented here are recently reported findings and perspectives related to bovine oocyte biology and analysis of blastocyst quality that addresses these concepts from a different angle supporting the complex nature of the very dynamic developmental window that encompasses late oogenesis up to blastocyst development. It was recently reported that the atypical nature of the oocyte is supported by extensive nurturing from the surrounding cumulus cells in the form of large cargo transfer as well as transfer of phosphocreatine as an alternate means of generating ATP to fulfill the oocyte's needs during the energy demanding process of maturation. It has been shown many times over that the determinants of early embryogenesis are embedded in the oocyte, however, transcriptome analysis dissociates embryonic yield from the concept of embryonic quality. Within the divergent gene expression, long non-coding RNAs represent a very functionally diverse class of transcripts that have yet been characterized. Taken together, it is clear that a clearer definition of both oocyte and embryonic quality are still needed to support the improvement of *in vitro* embryo production.

Keywords: embryo quality, oocyte competence, RNA transfer.

Introduction

Reproductive success can be broadly summarized as the birth of a viable and healthy offspring. This achievement relies on the completion of numerous complex and selective developmental steps occurring throughout the reproductive process. Interestingly, very few gametes ever get to contribute to the next generation, and reproductive success relies heavily on the quality of those gametes. Recent findings support an added contribution from the male gamete through the transfer of proteins (Saunders *et al.,* 2002) and RNA (Ostermeier *et al.,* 2004) at fertilization and also of an epigenetic legacy (Lambrot *et al.,* 2013) but the constitution of the early embryo is dependent upon the constitution of the egg. In addition to the maternal genome, the oocyte also provides the cytoplasmic components including RNA and protein reserves as well as the mitochondrial contingent all of which are necessary to sustain early embryo development.

To achieve reproductive success, the oocyte must display competence to resume meiosis, to cleave upon fertilization, to sustain early development (namely to activate its genome), to establish a pregnancy, and to sustain fetal growth and development until birth. It is well accepted that succeeding in the first events does not ensure the success of subsequent ones (Sirard *et al.,* 2006). It is this capacity to successfully complete these steps that is referred to as developmental competence. As it has already been discussed, developmental competence is ¨a convenient but biologically fuzzy concept¨ (Duranthon and Renard, 2001) since in its broadest sense, the impact of the oocyte is carried up to the birth of a healthy and fertile offspring. However, other factors, excluding the oocyte, have to be considered such as the reciprocal interaction between the conceptus and the endometrium in the establishment of a pregnancy. Generally, a narrower definition is used where the oocyte's intrinsic developmental competence is studied up until the blastocyst stage after which the requirement for the uterine environment becomes a confounding effect.

By comparing blastocyst rates when producing embryos either *in vitro* or *in vivo* there are three main processes undoubtedly affecting developmental outcomes; they are oocyte maturation, fertilization, and embryo culture. It has been shown that this shorter view of developmental competence is heavily influenced by the quality of the oocyte at the outset and completion of maturation (Rizos *et al.,* 2002). Still today, the characteristics defining oocyte quality are vague and subjective. Studies have focused on the morphology of the cloud of somatic cells surrounding the oocyte and the visual aspect of the gamete's cytoplasm (Blondin and Sirard, 1995). As a token of this, using these characteristics it is possible to choose a subset of cumulus-oocyte complexes (COCs) that will reach the stage of blastocyst *in vitro* in a greater proportion than the unselected population but the rates are seldom higher than 50% and some COCs that do not harbour

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the targeted criteria are able to produce a viable embryo.

In complement, it has been shown that the microenvironment to which the oocyte is submitted can have a profound impact on the proportion of oocytes reaching the blastocyst stage (Lequarre *et al.,* 2005). In order to improve *in vitro* embryonic yields, conditions such as oxygen tension (Quinn and Harlow, 1978; Olson and Seidel 2000; Correa *et al.* 2008) and media composition (Harper and Brackett, 1993a, b; Lonergan *et al.,* 1996; Baldoceda-Baldeon *et al.,* 2014) have been tested and shown to increase the number of blastocysts but still rarely over 50%. The bulk of these studies have been done using COCs aspirated from ovaries collected post-mortem. Using this source, it has been shown that collecting COCs immediately after death leads to low blastocyst rates whereas letting the ovaries incubate in warm saline for a few hours before COC aspiration improves blastocyst rates (Blondin *et al.,* 1997). The mechanism by which oocyte quality improves within the dying follicle is still unknown but it is again a good example of how the oocyte's microenvironment can influence the acquisition of developmental competence.

Considering the selected follicular sizes from these post-mortem ovaries are generally between 3 and 8 mm whereas a bovine preovulatory follicle can reach over 20 mm (Quirk *et al.,* 1986), the suboptimal embryo production could thus be associated with incomplete follicular growth or be representative of a situation where not all oocytes can acquire developmental competence. The latter was challenged when manipulation of the hormonal regimen during ovarian stimulation was shown to produce cohorts of immature oocytes capable of sustaining *in vitro* development to reach the blastocyst stage sometimes at a rate of 100% (Blondin *et al.,* 2002; Nivet *et al.,* 2012). Many reviews have already summarized these observations (Sirard *et al.,* 2006; Fair, 2010; Keefe *et al.,* 2015; Moussa *et al.,* 2015) but yet, the nature of the cues inducing the acquisition of developmental competence is unknown alike the distinctive characteristics harboured by a developmentally competent oocyte.

The need to understand and improve oocyte quality is fuelled by the application of assisted reproductive technologies (ART) both in human to palliate to infertility, and in livestock to increase the rate of selective breeding. In the field of *in vitro* embryo production (IVP) efforts have been directed towards the improvement of culture systems to increase blastocyst yields which have now lead to some concerns regarding the potential for these conditions to cause short and long term effects on embryo quality. These effects from culture conditions can be observed as: a shift in developmental kinetics (Holm *et al.,* 2002), a skew in male: female ratio (Kimura *et al.,* 2005, 2008), and lower tolerance to cryopreservation (Rizos *et al.,* 2003, 2008). It is also known that *in vitro* embryo metabolism varies according to culture conditions and differs from *in vivo* derived counterparts (Krisher *et al.*, 1999; Khurana and Niemann, 2000).

Concerns over the potential carry over impacts on the development of pathological phenotypes much later than the blastocyst stage were reported at the turn of the millennium. Embryo production *in vitro* was associated with the large/abnormal offspring syndrome in ruminants (Young *et al.*, 1998; McEvoy *et al.,* 2000) which was then paralleled in human with higher frequency of imprinted disorders namely the Beckwith-Wiedemann, Angelmann, Prader-Willi and Silver-Russell syndromes (reviewed by Jacob and Moley, 2005), in addition to higher abortion rate and higher fetal abnormality rate (Taverne *et al.*, 2002). Concerns over the long-term impact of ART now encompass all steps including the ovarian stimulation regimen (Denomme and Mann, 2012, 2013) for their potential extended effects on fetal development and even on disease development in adult life (Chen *et al.*, 2011; Hart and Norman 2013a, b; Chen *et al.*, 2014). These long-term impacts are believed to be carried by epigenetics and have so far been studied with most focus at the level of DNA methylation. These concerns bring forth the need to define embryonic quality and include a concept pertinent to embryonic health or "normalcy".

Genome wide gene expression has been used to describe embryos mostly as a mean to compare *in vitro* to *in vivo* produced counterparts. These studies have mainly been conducted with the perspective of increasing embryonic quality to increase developmental competence. Several recent reviews have evaluated the observations (Driver *et al.*, 2012; Gad *et al.*, 2012) and as expected, given a different environment, embryonic cells adapt their gene expression. The challenge remains to determine which genes or extent of gene expression are associated with embryonic quality or are rather deviances that will lead to poor phenotypes. Both the concepts of oocyte developmental competence and embryonic quality are closely intertwined and can also be distinct in nature. A better understanding of oocyte biology is necessary as a basis of defining what makes a "good" oocyte, alike a better understanding of embryogenesis is necessary to define the interval within which an embryo can be defined as "good". Recent observations add to the complexity of these concepts. We have used the bovine model for the potential application to produce more embryos from high genetic merit donor cows, as well as for its value as a model for human reproduction being a large mono-ovular mammal with similar follicular dynamics and kinetics of early embryogenesis.

Oocyte biology

It is known that oocyte developmental competence is acquired once the gamete reaches full size. In bovine, oocytes from <2 mm follicles have not reached full size and show little capacity to mature, whereas full size oocytes are found in 3 mm follicles

and develop to reach blastocyst stage at a rate around 30-35% (Motlik *et al.*, 1984; Fair *et al.*, 1995; Feng *et al.*, 2007). It is known that oogenesis is progressing in synchrony with folliculogenesis through the close interdependence on the bilateral communications occurring between the follicular cells and the gamete. The granulosa cells communicate through paracrine signalling (for example EGF-like peptides; Gilchrist, 2011), while the cumulus cells and more precisely the inner layers of the corona radiata which bear cellular extensions reaching through the zona pellucida (Fig. 1) communicate with the oocyte through the gap junctions found at the articulation between the projection's end and the oocyte plasma membrane.

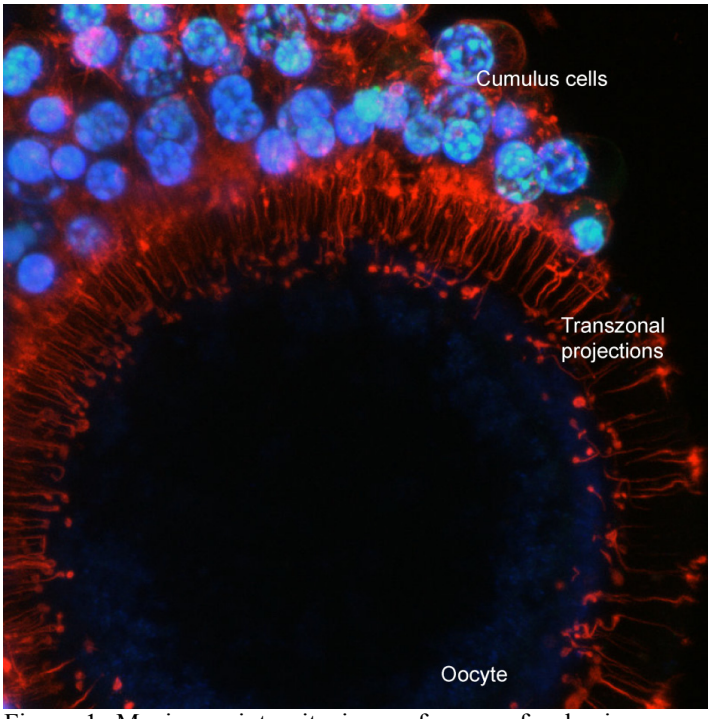

Figure 1. Maximum intensity image from confocal microscopy showing the transzonal projection. Numerous actin microfilaments in projections are coloured in red by the rhodamine phalloidin stain. Cumulus cells nuclei are stained in blue (Hoechst 33342).

Transferring cargo to the oocyte

Direct transfer from the somatic cell to the oocyte is known to occur where cyclic nucleotides control meiosis resumption. The importance of this communication has been the subject of recent reviews (Gilchrist, 2011). The use of gap junctions is also known to limit the size of transferred material to <1 kDa. This is unlike other animal models like *c. elegans* and *drosophila* where large material is delivered during oogenesis either from canals bridging oocytes together or from supporting nurse cells (Deng and Lin, 1997; Wolke *et al.*, 2007). We have recently reported that transzonal projections (TZPs) are very large channels made of actin filaments (Macaulay *et al.*, 2014). RNA granules were abundantly found within these structures and from a de novo labeling assay combined with a transcriptomic survey of the RNA found within the zona pellucida, it was proposed that cumulus cell transcripts are selectively and actively shuttled towards the oocyte. The transcriptome comprised of messenger RNAs as well as long non-coding RNAs (Macaulay *et al.*, 2014). Analysis of the interconnection showed that vesicles are secreted and contact is maintained until 9 h after initiation of maturation after which the connections detach and are completely broken at 22 h (Macaulay *et al.*, 2014). Comparison of transcriptomes before and after maturation allowed identification of mRNAs increasing in abundance in the gamete during maturation. The cumulus cells origin is supported by the known transcriptional silence occurring in the oocyte associated with chromatin condensation (Lodde *et al.*, 2008) in addition to the fact the increase was only observed in intact COCs not in denuded oocytes (Macaulay *et al.*, 2014). Large cargo transfer was further proven by reconstructing COCs; placing denuded oocytes into culture with transfected cumulus cells expressing a GFP fusion protein. Following reconstruction, both GFP coding transcripts and protein were detected in the oocyte (Macaulay *et al.*, 2014). From these results, it seems that the somatic cells continue to nurture the gamete even during its

transcriptionally quiescent period. The contribution of these transfers to oocyte quality and developmental competence remains to be established. Recent data indicates a subset of the transferred mRNAs could be translated and play a role in supporting maturation (Macaulay *et al*., 2015; Université Laval, Québec, Canada; manuscript submitted for publication). This opens a window of opportunity to influence oocyte quality through the cumulus cells. Additionally, it is known that early development relies on maternal stores of mRNAs where prior to genome activation, transcripts stabilized in ribonucleotide particles are recruited to support the protein synthesis demand (Sternlicht and Schultz ,1981). As such, cumulus cells could complement maternal reserves during the last stage of COC preparation.

Mitochondria and oocyte energy production

Cumulus cells are also known to support the oocyte by transferring energetic substrates such as lactate and pyruvate (Bilodeau-Goeseels, 2006). Typically, ATP production is sustained by glycolysis which produces pyruvate that is transported to the mitochondria to be metabolized by the Krebs cycle and the oxidative phosphorylation occurring in the cristae. However, the mammalian oocyte is an atypical cell where it was found that the glycolytic pathway is impaired missing the phosphofructokinase enzyme (Cetica *et al*., 2002). As an alternative, pyruvate is pumped from the cumulus cells into the oocyte where it is metabolized to generate ATP (Conti *et al.,* 2002; Bilodeau-Goeseels *et al.,* 2007). However, the extent of this energy production can be questioned since the gamete's mitochondrial contingent is odd displaying a round/ovoid form with few cristae and that can be hooded or contain a vacuole (Bavister and Squirrell, 2000). It is believed that the low potential for oxidative phosphorylation per mitochondrion is compensated for by their large number (250,000-300,000; reviewed by (Sutton-McDowall *et al*., 2010).

By exploring the transcriptome of polyribosomes during oocyte maturation, one main characteristic stood apart where adenylate kinases and creatine kinase where highly represented in this subpopulation of actively translated mRNAs (Scantland *et al*., 2011, 2014). This highlighted a potential for the adenosine salvage pathway to contribute to the ATP pool of the oocyte. This process needs two enzymatic reactions. The first one implicates the adenylate cyclase that catalyzes the interconversion of adenine nucleotides, and the second one is performed with the creatine kinase that catalyzes the conversion phosphocreatine and ADP to generate creatine and ATP (Fig. 2).

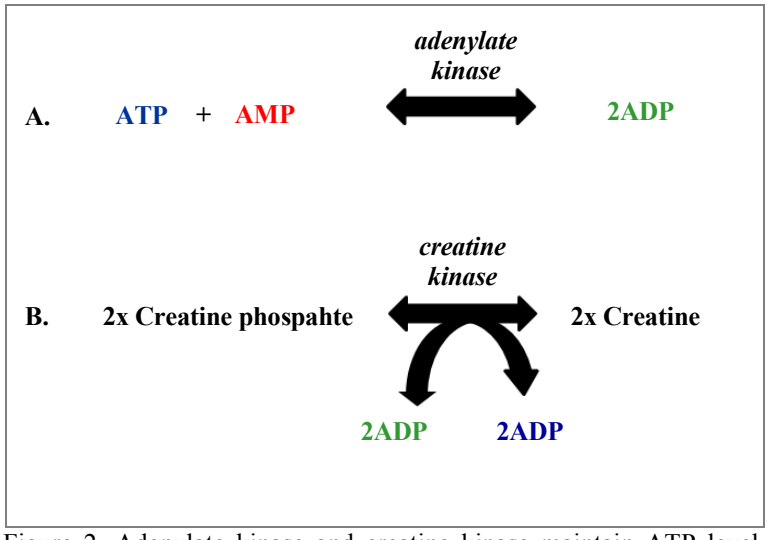

Figure 2. Adenylate kinase and creatine kinase maintain ATP level during oocyte maturation.

Through ATP content measurements during maturation and the addition of specific inhibitors and substrate, it was shown that the bovine oocyte is capable of phosphorylating AMP to produce ATP (Scantland *et al*., 2014). The proposed model is based on recycling the residuum generated by meiosis resumption. More precisely, the large quantity of cyclic AMP (cAMP) produced and transferred from the cumulus cells to the oocyte to control meiosis arrest is converted to AMP through the activity of phosphodiesterases when meiosis resumption is triggered leaving free AMP available for the adenosine salvage pathway. This alternative source of ATP may be important for oocyte quality to support protein synthesis and phosphorylation important for oocyte maturation (Stojkovic *et al*., 2001). The mitochondrial impact can act on the long term due to their numerous functions beyond energy production. Indeed, mitochondria also play a central role in other processes underlying oocyte quality and also developmental competence. The mitochondria have

important roles in fertilization (being involved in intracellular calcium storage) and embryo development (being the control center of programmed cell death; Santos *et al.*, 2006).

It is at the 4 to 16-cell stage that the mitochondria elongate and the numbers of transverse cristae increase alongside higher demand for glucose and pyruvate (Thompson *et al.*, 1996; Bavister and Squirrell, 2000; Wilding *et al.*, 2009). It was shown that reduced efficiency of mitochondrial respiration and ATP content in the oocyte and early embryo are correlated to a decrease in embryo development, and that maternal aging further influences this relationship (Stojkovic *et al.*, 2001; Nagano *et al.*, 2006; Wilding *et al.*, 2009). Furthermore, maternal ageing has been associated with less metabolically active mitochondria which may confer long term impacts since the offspring are at greater risk of developing obesity and other metabolic syndromes (Wilding, 2015). These observations exemplify how oocyte quality can influence not only developmental competence and embryonic quality but also convey far-reaching health effects that persist into adulthood.

Embryo quality

In order to increase blastocyst rates, culture conditions have been modified extensively by the addition of compounds that would scavenger free radicals or increase cell survival by adding anti-apoptotic compounds (Brison and Schultz ,1997; Kolle *et al.*, 2002; Livingston *et al.*, 2004; Block *et al.*, 2008; Khalil *et al.*, 2013; Wang *et al.*, 2013) . Ultimately, the challenge remains to determine which embryos are "sound" for transfer. As such, different morphological characteristics are used to assess embryo quality such as developmental kinetics where faster cleavage rates (fast 2-cells) display better blastocyst rates similar to embryos displaying "normal" cell-cycle lengths or appreciating the embryonic structure looking for symmetrical cells, embryonic homogeneity, low fragmentation, less apoptotic cells, appropriate cell lineage distribution with the inner cell mass/ trophectoderm (ICM/TE) ratio, or cell to cell interaction where tighter gap junctions are preferred (Lee *et al.*, 1987; Van Soom *et al.*, 1997a, b; Holm *et al.*, 2002; Houghton *et al.*, 2002). These grading characteristics can be subjective, and variable results are often obtained in regard to their *in vivo* similarity or gestation success after transfer.

Consequences of *in vitro* culture systems

As mentioned, since the introduction of bovine *in vitro* embryo production, blastocyst rates have been improved through the use of more suitable environmental conditions including media composition and supplementation. However, embryo production from oocytes aspirated from ovaries collected at the local abattoir has reached somewhat of a plateau with 30 to 40% of oocytes developing into blastocysts. Improvement strategies have generally used blastocyst rates as the target metric where increased production of embryos equates to a better production system that is often also interpreted as one that produces better quality embryos. The observations of shifts in the sex ratio and the increased frequency of difficult parturition due to the offspring overgrowth raised questions regarding the impacts of culture conditions on embryonic quality. The presence of components from biological origins (serum, BSA, hormones) has been addressed, as their use is now known to represent an important source of variance due to contaminants that remain following purification (Kane, 1983; Batt and Miller, 1988; Rorie *et al.* 1994). For more than a decade, embryonic gene expression has been surveyed in order to determine the impacts of culture conditions as well as to attempt to provide a definition of embryonic quality (Wrenzycki *et al.*, 2007; Carter *et al.*, 2010; Clemente *et al.*, 2011). So far, it has been challenging to compare results between studies due to the use of distinct technological platforms, as well as the difficulty in interpreting results as at least two confounding effects arise from any modification to the embryonic culture medium where deviance in gene expression is part of the natural cellular response or perhaps a part of an ill fated response (For review see Robert, 2010; Robert *et al*., 2011). This is caused by the lack of a reference of quality where the comparison with *in vivo* collected embryos is somewhat inappropriate. Although it is the most natural reference, it is also expected that *in vitro* conditions should lead to the some deviance in gene expression since both types of embryos (*in vivo* and *in vitro*) are grown is a very different microenvironment. Thus, transcriptomic divergences are expected but to which extent these deviations become a sign of lower quality? Timing developmental stage between *in vivo* and *in vitro* is also a challenge since timing post-insemination differs from timing post-fertilization. It has also been challenging to delineate between treatment effects and deviance in gene expression caused by a shift in development kinetics leading to the comparison of blastocysts at different developmental stages or by a shift in sex ratio that would highlight the gender associated differences in gene expression (Bermejo-Alvarez *et al.*, 2010). These can become confounding factors but are also part of the treatment effect e.g. a treatment can induce a shift in developmental kinetics that will lead to the comparison of embryos at slightly different developmental stages (early blastocysts vs. expanded blastocysts); in such situation, comparing transcriptomes will identify deviations that may not be a sign that embryos are abnormal but may rather be representative that the embryonic cohorts are different.

In one of our studies, the embryonic transcriptome was compared across ten different *in vitro*

production systems (Table 1; Cote *et al.*, 2011). The initial premise was to collect embryos at a definite time post-fertilization, and compare blastocysts collected without selecting for embryonic morphology or gender as any skew in these factors is part of the treatment effect. Again, *in vitro* produced embryos have been compared to an *in vivo* embryos used as reference. Unexpected results showed that some treatments with poor embryonic yields generated transcriptomic signatures closer to the *in vivo* reference than the high yielding systems, and that these high yielding systems were characterized with very heterogeneous embryonic cohorts. To reconcile these observations we proposed that increasing embryonic rates necessitate more lenient conditions increasing developmental kinetics and allowing the survival of weaker embryos that would otherwise die. We raised the question that it may be necessary to eliminate the weak embryos to minimize the expression of poor phenotypes (Cote *et al.*, 2011). Although these embryos were not transferred, this comparative study suggests that it is possible to produce a lower number of better quality embryos therefore dissociating developmental competence defined as the maximum number of embryos produced from the concept of embryonic quality.

In a subsequent study, it was shown that when selecting a subset of blastocysts using a defined morphology at a fixed time post-fertilization, the impact of culture conditions on gene expression deviations are largely minimized (Plourde *et al*., 2012). This confirmed that previous findings are related to the structure of the embryonic cohort. This study also showed that the source of the oocytes either collected from abattoir ovaries or from ovum pick-up following an ovarian stimulation protocol has a more profound impact on blastocyst gene expression than the culture conditions (Plourde *et al*., 2012). This by contrast supports the close relationship that exists between oocyte quality and embryonic quality. In this study, it was also observed that one of the main impacts of *in vitro* embryo production across all treatments compared to *in vivo* blastocysts involved genes related to mitochondrial functions (Plourde *et al*., 2012). This again supports the theory that mitochondria play a central role in both oocyte quality and developmental competence. This is in accordance with the quiet embryo hypothesis where analysis of the metabolites from the culture media can be associated with embryo quality (Leese, 2012). This concept correlates the metabolism of individual preimplantation embryos to their subsequent viability where a low metabolism observed in regard to low glycolytic rate and amino acid turnover and their high antioxidant capacity characterize the best embryos (Leese, 2012). So, a healthy embryo will have "quiet" metabolism because it spend less energy to rectifying damage to the genome, transcriptome, and proteome, or has less injury compared to a non-viable embryo (Leese *et al.*, 2007).

Table 1. Comparison of ten different embryonic *in vitro* production systems.

Treatment no.	Oocyte maturation (IVM)	Embryo culture (IVC)	Blastocyst yield	Blastocyst morphology at Day 7	Distance of transcriptome from reference	Overall variance between replicates
1	*In vivo*	*In vivo*	N/A	N/A	Reference	++
2	SOF-BSA	SOF-BSA	+++	Fully expanded	++	+
3	SOF-serum	SOF-BSA	++	Fully expanded	++++	++
4	SOF-BSA	SOF-serum	+++	Fully expanded	+++	++++
5	SOF-serum	SOF-serum	+++	Fully expanded	++	++++
6	SOF-BSA	SOF-BSA *	+	Small and dark	+	+++
7	SOF	SOF	+	Small and dark	+++	++
8	SOF-BSA	SOF co-culture	+++	Fully expanded-hatching	++	++
9	TCM-serum	TCM co-culture	+++	Fully expanded-hatching	+	++
10	TCM-serum	SOF-BSA	+++	Expanded but darker	++++	++
11	TCM-serum	TCM-serum	+	Small and dark	+++	+

*Serum was added on the last 2 days of embryo culture and thus after embryonic genome activation in an attempt to determine whether fully active embryonic cells could better use this hormone-rich supplement. [+]Subjective appreciation of distance from reference and variance within treatment. Adapted from Cote *et al*., 2011.

Mining embryonic trancriptome: the role of the long non-coding RNA

During the transcriptomic surveys, it was found that bovine embryonic cells express a large contingent of uncharacterized transcripts (Cote *et al.*, 2011; Plourde *et al.,* 2012). Sequence analysis indicate that these transcripts do not contain an open-reading frame meeting the minimal international standard of 300 nucleotides in length (100 amino acids; Dinger *et al.*, 2009) and that they are generated from both the nuclear and the mitochondrial genome (Cote *et al.*, 2011; Plourde *et al.*, 2012). These long non-coding RNA (lncRNA) constitute the largest class of non-coding RNA. By definition, they are longer than 200 nucleotides, and do not serve as a template for protein synthesis. Thus far, approximately 15,000 human lncRNA genes have been identified (Derrien *et al.*,

2012). Evidence demonstrating that they have various biological functions and play critical roles in the embryonic stem cell regulatory circuit began to emerge through the mouse model only a few years ago (Guttman *et al.,* 2010; Sheik Mohamed *et al.,* 2010). However, understanding the role of lncRNA is challenging and only 1% of all human non-coding transcripts have been associated with a function (Perkel, 2013).

When comparing transcriptomes to identify differential gene expression associated with culture conditions, it was shown that some of these lncRNA molecules are highly responsive to the embryonic microenvironment (Cote *et al.*, 2011; Plourde *et al.*, 2012). So far, their roles have not been characterized. Since non-coding RNAs are part of epigenetics as some of them can regulate gene expression (Bouwland-Both *et al.*, 2013; Bhan and Mandal, 2014) it was anticipated that some of these lncRNAs could be indicative of epigenetic perturbations caused by the culture conditions (Le Bouc *et al.*, 2010; Santos *et al.*, 2010). To investigate this, three candidates have been further characterized and were found surprisingly in the cytoplasm rather than in the nucleus and also to be associated with the polyribosomes (Caballero *et al.*, 2014). Moreover, one of them was found amongst the transcripts isolated from the TZPs between cumulus cells and the oocyte (Caballero *et al.,* 2014). Knocking down this transcript in matured oocytes unexpectedly accelerated embryo developmental kinetics (Caballero *et al.,* 2014). By overlaying the transcriptome and DNA methylome analyses, the knock down was shown to impact on four concordant genes (Coxsackie virus and adenovirus receptor (CXADR), advillin (AVIL), CD9 molecule, and plasminogen activator urokinase (PLAU)) where DNA methylation and transcript abundance follow the rule that increasing methylation results in lower gene expression and vice versa. The last two identified genes are known to be associated with the demise and the survival of blastocysts during the pre-implantation period, respectively (El-Sayed *et al.* 2006). The roles of these lncRNAs in early embryogenesis have yet been elucidated. Like the proteins from the mRNAs, it is possible that lncRNAs are involved in numerous aspects of cellular functions.

Conclusions

It is clear that both concepts of oocyte developmental competence and embryonic quality are closely related, evidenced by the possibility to increase the number of blastocysts produced *in vitro* by modulating follicular conditions. It is conversely true that it is possible to modulate culture conditions to increase the number of low quality blastocysts. Therefore, blastocyst rate may not be a sufficient metric to improve *in vitro* embryo production. As others have proposed for more than a decade it seems clear that a common denominator between embryonic developmental potential and quality is associated to the presence of healthy mitochondria (Van Blerkom, 2008, 2009, 2011). This is the basis of the therapeutic intervention done to increase fertility of the oocyte of aged women (St John, 2012; Tilly and Sinclair, 2013). Although promising any development in the field of embryo production still is in need for the definition of a good quality oocyte and of a top quality embryo. Given the impact of the epigenome and the sensitivities of the oocytes and early embryos to their surrounding environment, the broader definition of developmental competence must be all-encompasing when the goal of embryo production is to induce gestation with healthy offspring as the end result.

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Metabolism in the pre-implantation oocyte and embryo

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Abstract

An understanding of oocyte and embryo metabolism is critical to understanding and developing *in vitro* culture systems. In the last 60-70 years there has been a constant evolution in the way metabolism studies have been conducted. This includes a change from studying the metabolism of the oocyte alone *vs*. as a whole cumulus oocyte complex. The study of *in vivo* environments has lead to the creation of defined sequential culture systems, resulting in overcoming developmental blocks and improved embryo development. And techniques for studying metabolism have evolved from the use of radiolabelled isotopes to increasingly specific fluorescence probes and metabolomics, allowing for large, integrative profiles. Metabolism is a potential diagnostic for selecting the most likely embryos to implant. We envisage the future of metabolism will involve the ability to measure 'more-in-less' (more substrates, less volumes) and allow for a holistic approach to understanding the relationship between metabolism and developmental competence, as it is unconceivable that a single metabolic output will be able to assess health and/or quality.

Keywords: embryo, *in vitro* embryo production, metabolism, oocyte.

Introduction

Fifty years ago Robert Edwards discovered that mechanical release of an oocyte from the ovarian antral follicle could initiate the final stages of oocyte maturation (Edwards, 1965). Since then, *in vitro* oocyte maturation (IVM), *in vitro* fertilisation (IVF) and culture of embryos post-fertilisation (*in vitro* embryo culture, IVC); collectively known as *in vitro* embryo production (IVP), has been widely utilised for the study of pre-implantation oocyte and embryo development and is increasingly utilised in livestock animal production and human assisted reproduction.

An understanding of the metabolism of cumulus oocyte complexes (COCs) and embryos is critical, not only to enable the creation of improved culture systems, resulting in the development of healthier *in vitro* produced embryos, but metabolism is a potential marker of developmental competence, determining which embryos are the healthiest and thereby have the highest chance of implantation and a healthy pregnancy.

There are numerous excellent review articles covering metabolism of the COC (Sutton *et al.*, 2003b; Thompson *et al.*, 2007, 2014; Sutton-McDowall *et al.*, 2010; Krisher, 2013) and the embryo (Bavister, 1995; Thompson, 2000; Leese *et al.*, 2008; Leese, 2012). With this in mind, the focus of this review is to present a brief synopsis of changes in pre-implantation metabolism through development, limitations to the current metabolic diagnostics used and possible future directions for determining metabolism of COCs and pre-implantation embryos. Furthermore, while we acknowledge that the COC and embryo utilise many energy sources such as lipids (Sturmey *et al.*, 2009; Dunning *et al.*, 2014) and amino acids (Wale and Gardner, 2012), this review will focus on the metabolism of carbohydrates and downstream signalling molecules.

Metabolism: timing (and stage) is everything

The peri-conception period, covering the final stages of oocyte maturation through to pre-implantation embryo development, is a highly dynamic period, with the COC and pre-implantation embryo exposed to several different micro-environments, ranging from the highly vascular, hence highly perfused ovarian follicle to the low oxygen levels (Tervit *et al.*, 1972; Maas *et al.*, 1976; Fischer and Bavister, 1993) and more mucus environment of the uterus. It is well established that the metabolism of the COC and pre-implantation embryo varies (Fig. 1) and this is largely reflective of the *in vivo* environment (Krisher, 2013).

In an attempt to improve IVP success, culture systems have been formulated based on the composition of the *in vivo* environment (reviewed by Summers and Biggers, 2003; Sutton *et al.*, 2003a; Table 1), resulting in significantly higher rates of developmental competence and pregnancy success rates. Indeed, pioneering work by Tervit and colleagues used the composition of sheep oviductal fluid (characterised by Restall and Wales, 1966) to create synthetic oviductal fluid (SOF) and performed culture in low oxygen concentrations (Tervit *et al.,* 1972), a system that is still widely utilised, with modified versions used throughout

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IVP in larger animals (Gandhi *et al.*, 2000).

However, due to the static, yet highly chemically defined nature of culture systems, *vs*. the highly perfused and complex environments *in vivo*, there is room for improvement and consequently the compositions of IVP media suites are constantly evolving. To date, the most successful media suites include sequential media to accommodate changing metabolic needs (Summers and Biggers, 2003; Lane and Gardner, 2007), although this is challenged within the human IVF field, suggesting that single media systems are suitable (Cohen *et al.*, 2008; Paternot *et al.*, 2010).

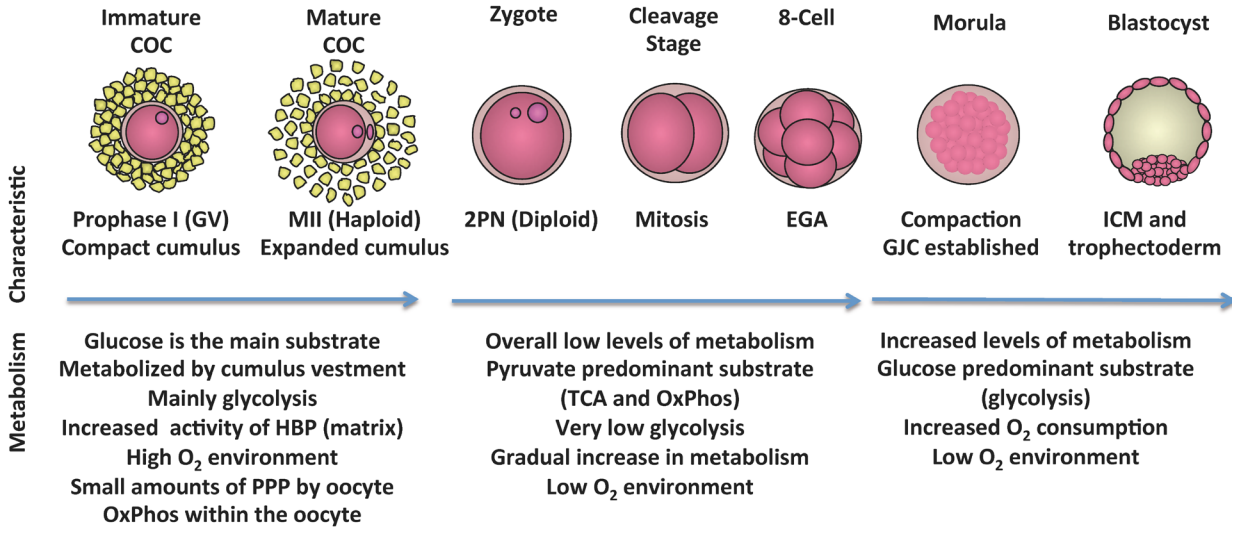

Figure 1. Changes in the metabolism of cumulus oocyte complexes (COCs) and preimplantation embryos. 2PN = 2 pronuclei; GJC = gap junction communication; GV = germinal vesicle; HBP = hexosamine biosynthetic pathway; ICM = inner cell mass; OxPhos = oxidative phosphorylation and TCA cycle = tricarboxylic acid cycle.

Table 1. Carbohydrate composition of the *in vivo vs*. *in vitro* environments that cumulus oocyte complexes (COCs) and embryos are exposed to.

	COC		Fertilisation		Embryo		
	In vivo	*In vitro*	*In vivo* (Oviduct)	*In vitro*	*In vivo* (Uterus)	*In vitro* (Cleavage)	*In vitro* (Post-Compaction)
Glucose (mM)	1.4-2.3[1] 2-3.8 [2]	1.5 (SOFM) 5.6 (M199)	2.4-3[3] 0.5-3.11[4]	2.8 (HTF) 0 (Fert TALP)	0.5[5] 0.02-0.04[6] 3.15 [4]	1.5 (SOFC1) 0.5 (G1.2)	3 (SOFC2) 3.2 (G2.2)
Lactate (mM)	3-6.4[1] 5-14.4[2]		2.5[7] 4.9-10.5[4]	21.4 (HTF) 10 (Fert TALP)	8.6[5] 5.9[4]	10.5 (G1.2)	5.9 (G2.2)
Pyruvate (mM)	0.4 [1]	0.33 (SOFM) 0.2 (M199)	0.2[7] 0.24[4]	0.33 (SOFM) 0.3 (HTF) 0.2 (Fert TALP)	0.17[5] 0.1[4]	0.33 (SOFC1) 0.32 (G1.2)	0.33 (SOFC1) 0.1 (G2.2)

SOF = Synthetic Oviductal Fluid; HTF = Human Tubal Fluid (Quinn *et al.,* 1985); Fert TALP = Modified Tyrode's Medium (Gardner *et al.,* 2004); G1.2/G2.2 (Lane *et al*., 2003). [1]Sutton-McDowall *et al.,* 2005; [2]Leroy *et al.,* 2004; [3]Lippes *et al.,* 1972; [4]Gardner *et al.,* 1996; [5]Dickens *et al.*, 1995; [6]Carlson *et al.*, 1970 and [7]Lopata *et al.,* 1976.

Pre-ovulation: the cumulus oocyte complex

Historically, the carbohydrate metabolism of the oocyte has been described (Biggers *et al.*, 1967; Rieger and Loskutoff, 1994; Bavister, 1995; Krisher and Bavister, 1999; Spindler *et al.*, 2000). However, in the last decade, the importance of the cumulus cells supplying the oocyte with nutrients and substrates to achieve developmental competence has emerged (Dumesic *et al.*, 2015), as a consequence of understanding the importance of the bi-directional communication between the oocyte and cumulus vestment (Eppig, 1991; Albertini *et al.*, 2001; Matzuk *et al.*, 2002). Thus, characterisation of the metabolic profile of the COC as a whole is essential in our view. However, the COC contains two distinct cell types with

different metabolic profiles: the oocyte predominantly undergoes oxidative phosphorylation and the cumulus vestment has a high rate of glycolytic activity (Thompson *et al.,* 2007). The primary substrate of the COC is glucose and is metabolised via numerous pathways to provide energy and substrates for extracellular matrix formation and cumulus mucification, nucleic acid synthesis and plays a major role as a stress/fuel sensing molecule (reviewed by Sutton-McDowall *et al.,* 2010). With the progression of COC maturation, metabolism increases steadily, with increases in glucose, pyruvate and oxygen consumption observed (Sutton *et al.,* 2003a).

The environment in which a COC is exposed to during maturation, both *in vivo* and *in vitro*, largely impacts its developmental competence (Sutton *et al.*, 2003c; Krisher, 2013; Dumesic *et al.,* 2015). For example, maternal hyperglycaemia and hyperlipidemia compromise COC health, embryo development and pregnancy outcomes (Chang *et al.*, 2005; Leroy *et al.*, 2008; Robker, 2008; Purcell and Moley, 2011; Van Hoeck *et al.*, 2011). To date, the technology to measure the metabolism of oocytes and COCs within the ovarian follicle does not exist, with measurements performed *ex vivo* and usually with some degree of further *in vitro* manipulation. This includes physical removal from the follicle, exposure to culture media, sometimes combined with hyperstimulation to retrieve adequate numbers of COCs. This begs the question as to the influence of even brief exposure to *in vitro* conditions on the metabolism of *in vivo* derived COCs. We have reported that even a brief exposure (1 h) of immature mouse COCs to "collection" media containing different concentrations of glucose can have a dramatic effect on post-fertilisation embryo development (Frank *et al.*, 2013). Aspiring to determine the precise differences between the metabolism of *in vivo* and *in vitro* matured COCs is not possible, as *in vivo* derived COCs must be removed to measure their metabolism.

Over the past decade, improvements in IVP success have largely been attributed to improved IVM culture systems, by creating environments that more closely mimic *in vivo* conditions. Systems that are more *in vivo*-like provide clues as to which metabolic parameters are associated with improved developmental competence; these are emerging from studies with media additives that improve COC development. An example is the addition of exogenous oocyte secreted factors (OSF), specifically recombinant bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), resulting in improved developmental competence (Gilchrist and Thompson, 2007). While OSFs promote the distinct cumulus cell phenotype such as mucification and proliferation (Buccione *et al.*, 1990; Salustri *et al.*, 1990a, b); steriodogenesis (Vanderhyden and Macdonald, 1998) and prevention of cumulus cell apoptosis (Hussein *et al.*, 2005), OSF also promote cumulus cell metabolism, as both glycolysis and *de novo* cholesterol biosynthesis is compromised within cumulus cells of oocytectomised complexes (OOX, a COC in which the oocyte is surgically removed). The activity of these pathways can be restored with the addition of exogenous OSFs (Sugiura and Eppig, 2005).

The complex nature of COC metabolism associated with enhanced developmental competence is well demonstrated by examining the impact of BMP15 and FSH supplementation *in vitro*. In the absence of FSH, cattle COCs treated with BMP15 alone consume less glucose and produce less lactate compared to FSH treatment alone, this is a predictable consequence of little cumulus expansion compared to standard IVM conditions, which utilize FSH. Yet both groups have similar rates of glycolytic activity (Sutton-McDowall *et al.*, 2012). Within the oocyte, BMP15 treatment promotes oxidative phosphorylation and tricarboxylic acid (TCA) cycle activity (FAD and NAD(P)H, respectively) and as a consequence, higher levels of antioxidants (reduced glutathione, GSH) and reactive oxygen species levels (ROS, H_2O_2; Sutton-McDowall *et al.,* 2012, 2015; Sudiman *et al.*, 2014) were detected. In comparison, FSH stimulates glucose consumption by cumulus cells, with increasing levels of glucose utilised via the hexosamine biosynthetic pathway for cumulus expansion towards the end of IVM (Sutton-McDowall *et al.*, 2005). Significantly, both these independent treatments improved developmental competence. Hence, BMP15 and FSH promote distinct metabolic pathways within the different compartments of the COC. When combined, FSH and BMP15 stimulate a metabolic equilibrium (Sutton-McDowall *et al.,* 2012, 2015), in which the metabolic effect of each was "masked", yet this combined treatment yielded the highest developmental competence (blastocyst rates).

Metabolism pre- and post-compaction

The first stage of oocyte-embryo transition is oocyte activation following sperm penetration. This includes the cortical granule reaction and hardening of the zona pellucida to prevent polyspermy, resumption of meiosis, pronuclear formation and syngamy. These events are initiated by cytoplasmic release of small signalling ions such as calcium and zinc (Wang and Machaty, 2013; Que *et al.*, 2014), with minimal gene transcript and energy demand. Zygotes and cleavage-staged embryos rely on the oxidation of carboxylic acids such as pyruvate and lactate via the TCA cycle and oxidative phosphorylation within the mitochondria, with minimal glycolytic activity as the demand for ATP is low (Fig. 1; Thompson, 2000). Post-compaction, in morula and blastocyst stage embryos, overall metabolism increases, with glycolysis becoming the predominant source of ATP, a pattern seen in mouse (Houghton *et al.*, 1996), cow (Thompson *et al.*, 1996), pig (Swain *et al.*, 2001; Sturmey and Leese, 2003) and

human (Gott *et al.*, 1990) embryos. In addition, oxygen consumption, TCA cycle and oxidative phosphorylation also increase (Thompson, 2000).

Development of improved embryo culture systems was driven by the inability to overcome the specific cell-cycle developmental block induced by an unsupportive culture environment. Early development in the presence of high levels of glucose and substrates results in Crabtree-like metabolism (increased glycolytic activity and depression of oxidative phosphorylation). Such conditions induce a developmental block coinciding with embryonic genome activation; namely a 2-cell block in mouse (Lawitts and Biggers, 1991) and at the 8-cell stage in ruminants (Thompson *et al.*, 1992; Gardner *et al.*, 1997; Summers and Biggers, 2003). As mentioned previously, the development of sequential culture systems, adapted to reflect the metabolic needs of COCs and embryos (i.e. reduced substrate concentrations in the pre-compaction period), has resulted in significant improvements in the developmental outcomes of IVP embryos, overcoming the developmental blocks.

How to measure metabolism

Metabolism can be measured in two ways, either direct measurement of metabolites (including associated proteins, genes or signalling molecules) within the COC and embryo, or sampling the surrounding environment, such as *in vivo* fluids or the culture media. Sampling of the *in vivo* environment has been critical in formulating culture systems based on the metabolic profiles of COCs and embryos and has resulted in improved embryo development (Summers and Biggers, 2003).

Direct measures within the COC, oocyte or embryo

PCR (mRNA), western blots (protein levels and post-translation modifications), direct enzyme assays and immunohistochemistry (localisation) have been used to study the presence and relative activities of key metabolic enzymes and downstream targets. However, a large proportion of the initial metabolism experiments were performed using radiolabelled substrates. The Hanging Drop assay involves culturing oocytes or embryos in ~3 µl of culture media containing cold and hot (radiolabelled) substrates. This drop was suspended in the lid of a centrifuge tube (or similar vessel) containing a solution of sodium hydroxide or sodium bicarbonate (the latter requiring CO_2 gassing), which acts as an isotope "trap" and provides humidification of the chamber (O'Fallon and Wright, 1986). Depending on which carbon/hydrogen was labelled, the production of labelled CO_2 or H_2O indicated the proportion of the substrate metabolised via particular pathways. For example, the production of $^{14}CO_2$ from [1-^{14}C] glucose measured activity through pentose phosphate pathway (PPP) and TCA cycle. Likewise, the production of ^3H_2O from [5-^{3}H] glucose is indicative of glycolytic activity. A summary diagram of the metabolism of labelled glucose isotopes is available in Downs and Utecht (1999).

Widely used in the 1980s-1990s (O'Fallon and Wright, 1986; Rieger and Guay, 1988; Downs and Utecht, 1999), the advantages of the Hanging Drop method included the radiolabelled products amplifying the metabolic signal, resulting in high sensitivity and the ability to measure metabolic pathway activity in single oocytes and embryos (Bavister, 1987). Classed as non-invasive, embryo transfers could be performed at the completion of the assay period (O'Fallon and Wright, 1986). However, this assay could not be used in conjunction with embryo transfer in human embryos due to the use of radiolabelled substrates. Furthermore, the availability of commercially available assays that allows absolute concentrations of substrates to be determined has increased. Examples of commercially available kits include ADP/ATP kits (Sutton-McDowall *et al.,* 2012; Zeng *et al.*, 2013; Richani *et al.*, 2014) or clinical chemical analysers for pyruvate, lactate and glucose.

The influence of metabolism on development can be studied using inhibitors and/or stimulators of specific enzymes within metabolic pathways. Oocytes and embryos are cultured in the presence of the antagonists/agonists and outputs such as nuclear maturation and developmental stage would then be assessed (Downs, 1997; Downs and Mastropolo, 1997; Downs *et al.*, 1998; Downs and Utecht, 1999; Sutton-McDowall *et al.*, 2006). In combination with other measurements of metabolism such as substrate turnover, the use of antagonists and agonists remains highly valuable in determining the impact of a metabolic pathway on oocyte and/or embryo competence.

More recently, the development of a variety of effective fluorescent probes that react with specific enzymes or substrate, combined with improved accessibility to confocal microscopy technology has improved the measurement of the metabolism at the single oocyte and embryo level as it has the capacity to combine information on quantification and localisation of activity. Unlike traditional labelling, such as immunohistochemistry, where cells need to undergo extensive processing, such as fixation and permeabilisation, a large proportion of these newer probes are designed for use in live cells. For example, glucose uptake into a COC can be measured using 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG), a fluorescent glucose analogue that is non hydrolyzable (Sutton-McDowall *et al.,* 2010; Wang *et al.*, 2012a, b), and this method of studying glucose uptake complements measures of expression of glucose transporter genes (Wang *et al.,* 2012a, b).

Improved and increased accessibility to

commercially available probes has been particularly advantageous to the study of mitochondria. Since mitochondrial health and functionality is dependent on multiple factors such as density, localisation and distribution, maturity and activity (Babayev and Seli, 2015), the following paragraphs will use mitochondrial labelling as an example of how probes target different characteristics.

The most commonly used mitochondrial probes are JC-1 and Mitotracker probes. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) is a dual emission, ratio-metric probe that has been utilized to measurement changes in mitochondrial membrane potential ($\Delta\psi_m$) in live mouse and human oocytes (Diaz *et al.*, 1999; Wilding *et al.*, 2001; Van Blerkom *et al.*, 2002, 2003; Zeng *et al.,* 2013). When $\Delta\psi_m$ is low, JC-1 exists as a monomer (green emission) and is converted to J-aggregates/dimers (red emission) with high $\Delta\psi_m$. Hence, the ratio of red to green fluorescence indicates changes in $\Delta\psi_m$ independent of mitochondrial size, shape and density. However, JC-1 has disadvantages, as it is very sensitive to concentration; with the use of too high JC-1 concentrations leading to false positives, is highly sensitive to other factors such as H_2O_2, requires a long incubation time and has poor cell retention (Perry *et al.*, 2011). While JC-1 works well in rodent oocytes and embryos, in our experience JC-1 has poor cellular permeability when incubated with cattle oocytes and embryos, requiring cell permeabilisation or removal of the zona pellucida; both processes may harm an oocyte and embryo, and therefore not favourable considering the probe is assessing cell function.

Alternatives to JC-1 are the Mitotracker range of probes: mildly thiol-reactive chloromethyl moieties that are lipophilic cations, hence are highly cell permeable and only fluoresce within cells. Furthermore, they are more robust than JC-1, with higher photostability, require less reaction time, have higher cell retainability and less cross-reactivity with other factors (Perry *et al.,* 2011). There are two main forms of Mitotracker probes; carboryanine or rosamine based. The fluorescence of carboryanine base probes, such as Mitotracker Green FM (MTG) are independent of $\Delta\psi_m$, hence indicators of total mitochondrial mass in combination with localization, particularly useful in studies comparing mitochondrial biosynthesis in immature *vs*. mature oocytes (Stojkovic *et al.*, 2001; Sun *et al.*, 2001; Sturmey *et al.*, 2006; Gendelman and Roth, 2012). In comparison, rosamine based probes, such as Mitotracker CMXRos, (MTR) are oxidized within cells and sequestered within the mitochondria, hence indicators of $\Delta\psi_m$ and activity (Castaneda *et al.*, 2013; Viet Linh *et al.*, 2013; Niu *et al.*, 2015; Sanchez *et al.*, 2015; Sutton-McDowall *et al.,* 2015). In a similar concept to JC-1, cells can be co-labelled with MTR and MTG to determine a ratio of active to total mitochondria (Pendergrass *et al.*, 2004), although to our knowledge, such a comparison has not been performed in oocytes or embryos.

With advancements in probe design, microscopy and imaging technology, image analyses has also evolved to measure different pixel attributes, such as distribution, co-localization and patterning, in addition to pixel intensity. This can improve the quality of information about the role of mitochondria under different states of oocyte and embryo health. Ultrasound sonography, dermatology and cancer research are fields that routinely use advanced imaging matrices to assess variations in patterns of pixel characteristics such as wrinkles, smoothness, uniformity and entropy (Castellano *et al.*, 2004; Alvarenga *et al.*, 2007; Mittra and Parekh, 2011) of images. In comparison, image analysis within the pre-implantation research field is largely limited to measurements of fluorescence intensity or visual assessment. We have recently utilized texture analyses (Haralick *et al.*, 1973; Murata *et al.*, 2001; Cabrera, 2006) to assess the influence of exposing cattle COCs to FSH and BMP15 on the distribution of MTR, monochlorobimane (MCB; indicative of reduced glutathione) and peroxyfluor 1 (PF1; measures levels of H_2O_2, a derivative of reactive oxygen species; Sutton-McDowall *et al.,* 2015). In addition to pixel intensity, textural analyses demonstrated an association with homogeneous localization of fluorescence with improved developmental competence (Sutton-McDowall *et al.,* 2015). As technology improves, the mechanisms through which outputs are measured will continue to evolve.

While fluorescent probe are of value to the study of metabolism, label-free and non-toxic methods for characterising metabolism and viability would be preferable, in particular as a potential diagnostic of oocyte and embryo health. Electron donors NADPH/NADH (NAD(P)H) and the electron acceptor FAD are endogenous fluorophores with different spectral properties and therefore can be measured simultaneously by confocal microscopy. NADH has both cytoplasmic and mitochondrial localisation, whereas FAD is exclusively localised to the mitochondria (Table 2). FAD and NAD(P)H are critical for energy homeostasis, hence measurement of levels indicates the redox state of cells (FAD: NAD(P)H; Skala and Ramanujam, 2010). Measurement of intra cellular autofluorescence has not been widely exploited for investigations into cellular metabolism of embryos. However, Dumollard *et al.*, 2007a, b) utilised autofluorescence as a method for label-free localisation of mitochondria (Dumollard *et al.,* 2007a) and to study the influence of energy substrates on redox state over time (Dumollard *et al.,* 2007b). Furthermore, autofluorescence measurements have demonstrated changes in redox ratios in COCs following IVM in the presence of OSF (Sutton-McDowall *et al.*, 2012, 2015; Sugimura *et al.*, 2014) and EGF-like peptides (Richani *et al.,* 2014).

Table 2. Parameters of autofluorescence molecules involved in metabolism.

	Electron	Localisation	Pathways	Excitation (nm)	Emission (nm)
NADH	Donor	Cytoplasm Mitochondria	Glycolysis TCA cycle Oxidative Phosphorylation	350	460
NADPH	Donor	Cytoplasm	PPP	350	460
FAD	Accepter	Mitochondria	Oxidative Phosphorylation	450	535

Sampling of the culture media

Standard techniques for measuring metabolites include mass spectrometry/chromatography and clinical chemical analysers (Sutton-McDowall *et al.,* 2012, 2014). Leese and colleagues devised fluorometric assays for measuring nano and pico litres of samples based on the oxidation and/or reduction of autofluorescence signalling molecules such as FAD and NAD(P)H (Leese and Bronk, 1972). Indeed, many of these assays are still used due to their high sensitivity and the ability to measure the metabolite turnover of a single COC and embryo.

Metabolomics is the newest member of the "omics" family and unlike other metabolic assays, brings a more holistic approach to profiles, as it allows not only measurement of substrate turnover but also changes in pathway activity and downstream targets (Krisher *et al.*, 2015). Metabolomics combines two technologies to separate (gas chromatography or high performance liquid chromatography) and detect (mass spectrometry, nuclear magnetic resonance or Raman spectrometry) larger numbers of metabolites within spent culture media compared to fluorometric assays and other analytical methods. Both quantitative or qualitative measurements can be performed with quantitative measures requiring the generation of standard curves, which limits the number of substrates that can be measured (Thompson *et al.,* 2014). Successful application of some metabolomics platforms for spent media analysis to measure embryo quality were initially favourable and indeed still pursued (Krisher *et al.,* 2015), but has since been abandoned for use in human IVF, as results were inconsistent and dependent on media formulations.

The future for metabolic measurement of oocytes and embryos

A massive knowledge gap remains in characterising the metabolome of COCs and embryos *in vivo* as the ability to measure this *in situ* is essentially non-existent. There is a need to create new technologies that allow for *in vivo* measurement of biochemical reactions, given that even short exposures to *in vitro* conditions can alter COC and embryo metabolism. The development of remote sensing diagnostics, such as micro optical fibres and nano-particles are options for remote sensing with minimal invasion. An ideal candidate is the adaptions of multiphoton endoscopes to micro-optical fibres to allow for *in vivo* measurement of autofluorescence, hence redox state of COCs and embryos (Helmchen, 2002).

Even *in vitro*, the metabolic requirements of COCs are dynamic, with high levels of plasticity, where as most measurements are taken at a single time point. Furthermore, numerous metabolic pathways are in play and differential activity can result in numerous downstream consequences. For this reason, the use of single measurements of single metabolic outputs is not sufficient. Platforms that allow multi sampling of different aspects of metabolism are critical for advancing our knowledge of COC maturation. This could be achieved using label-free technologies and non-toxic, reversible probes, allowing for repeated measurements and changes in metabolism, crucial for dynamic periods in development such as oocyte maturation, fertilisation and embryonic genome activation. Essentially measuring more in less. A long-term goal could involve the development of sensing probes and systems that could be integrated into incubators, allowing the constant monitoring of changes in metabolism and thereby predict oocyte and embryo health and quality.

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What is new in the cryopreservation of embryos?

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Abstract

Embryo cryopreservation represents a pivotal tool for the long-term storage and exchange of valuable genetic resources of livestock and endangered species. The innumerable applications of embryo cryopreservation in human medicine, animal production, as well as in other embryo biotechnologies for research purposes are calling for standardized protocols that can be used in these different fields. This review will provide the reader with a brief outline to "the classics" of embryo cryopreservation procedures in farm animals and with a deeper insight into "the new trends". Moreover, the cryopreservation effects on the embryo will be revised; from the easily visible cellular damage to the damage at the transcriptomic, proteomic and lipidomic level, and fresh attention will be given to the epigenetic effects of this technology. Finally, we will go through personal considerations to take into account when embryo cryopreservation is used: how to select the best embryos for cryopreservation, the eternal question for how long can we store the cryopreserved embryos, the fact that size and fat matter when it comes to embryo cryopreservation and ultimately, our suggestion about designing cryopreservation protocols "*à la carte*" attending the needs of each type of embryo.

Keywords: cryopreservation, embryo, livestock animals, slow freezing, vitrification.

Introduction

Embryo cryopreservation has been a very useful tool for embryology since the first successful cryopreservation of mouse embryos in 1972 (Whittingham *et al*., 1972). This technology is the best method for the long-term preservation of valuable genetic resources from experimental and livestock animals. The use of cryopreservation is also essential for the widespread use of embryo transfer, which allows the exchange of genetics with reduced transportation cost, avoiding animal welfare problems and with a minimal risk of disease transmission. At present, millions of offspring have been born from cryopreserved embryos of more than 40 mammalian species (Saragusty and Arav, 2011).

The improvement of freezing protocols and the development of the vitrification technique have led to great advances in embryo cryopreservation over the last thirty years. Comparing current results with those obtained when the first freezing systems became available, it is evident that we are on the right path. Cryobiologist and reproductive biologists have provided with a better understanding of the physical principals of cryopreservation techniques (Liu *et al*., 2012) and their short- and long-term biological effects on the embryo. Today embryo cryopreservation is routinely used in bovine commercial embryo transfer (ET) programs. According to the 23rd annual report of International Embryo Transfer Society (IETS) of the data collected during 2014 for embryo transfer (ET) activity 2013, almost 60% of the transfers of in vivo derived bovine embryos were performed with cryopreserved embryos with high variations between countries (IETS, 2014). For other domestic animal species (small ruminants, pig, and equine) little is known, mainly due to the lack of reporting activity for these species. In addition, the difficulty and high cost of obtaining large numbers of embryos in these species has limited the number of cryopreservation studies performing ET. However, it is believed that ET activity of cryopreserved embryos is increasing, mainly in pigs.

To date, the absence of a perfect universal protocol and the low survival and farrowing rates obtained using slow freezing in some mammalian species (reviewed in Vajta, 2013) represent the major hurdles for a more widespread use of embryo cryopreservation.

In this review we will present the latest advancements achieved in embryo cryopreservation and some of the big challenges that cryobiologists and reproductive biologists need to overcome in the next years. Our purpose is to give some hints that can serve researchers as a guide for optimizing embryo cryopreservation protocols that can be routinely used in a wide range of species.

Strategies and their principles: slow freezing and vitrification

Two basic strategies have ruled the embryo cryopreservation field: the traditional slow freezing, also referred as conventional "equilibrium freezing" or "controlled slow freezing" and vitrification. In the

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present review we briefly mention the basic principles of these two strategies, since further detailed reviews on these systems can be found elsewhere (Leibo and Songsasen, 2002; Mazur, 2004; Fahy and Rall, 2007).

In the slow freezing process, the embryo is placed in a hypertonic solution containing 1-1.5 mol/L low molecular weight permeable cryoprotectants to facilitate partial embryo dehydration and therefore to avoid intracellular ice crystal formation during cryopreservation. Embryos are slowly cooled (0.2-2.0ºC/min) using a programmable freezer to sub-zero temperatures (-30 to -70ºC), and then plugged intro liquid nitrogen (-196ºC). In this procedure, embryos reach osmotic equilibrium with the cryoprotectants (CPAs) solution before freezing (Palasz and Mapletoft, 1996; Youngs *et al*., 2011). The slow freezing procedure has proven effective for mice, cattle and human embryos. However, for those embryos more sensitive to chilling injury, such as the pig embryo, *in vitro* produced or early stage embryos the slow freezing is not efficient.

Vitrification is defined as the solidification of a water-based solution forming a glass-like amorphous vitreous state without ice crystal formation. Vitrification can be achieved by using high cooling rates and high concentration of CPAs (Fahy *et al*., 1984). Embryo vitrification that was first reported by Rall and Fahy (1985) has two main advantages compared to slow freezing; it eliminates ice formation and reduces chilling injury (reviewed in Kasai and Mukaida, 2004). Vitrification is more effective, much quicker and simpler than controlled slow freezing. Since it does not require computerized equipment, it can be done even under field conditions. However, it requires highly trained personnel for manipulating embryos in small volumes and in short equilibration times. In the vitrification process, the embryo is exposed first to a low CPAs concentration solution (1-1.5 mol/L) and then to a much more concentrated solution (4-8 mol/L). Vitrification relies especially on 2 aspects that are closely linked: 1) A very high cooling rate (around 20000 ºC/min or higher), which is achieved by plunging the sample in liquid nitrogen (-196ºC) and by using different devices or straws (reviewed in Arav, 2014) that allow embryo vitrification in minimal volumes; and 2) the high viscosity of vitrification media, which depends on the concentration of the CPAs. The high concentration of cryoprotectants needed to achieve high viscosity is the main concern of vitrification because it can be toxic for the embryo and may cause osmotic damage (Libermann *et al*., 2002). The cooling rate and the concentrations of CPAs are inversely related. The faster cooling is undertaken, the lower CPAs concentration is necessary to achieve vitrification (Liu *et al*., 2012). This is a key point to reduce toxicity of vitrification solutions.

Important aspects of successful cryopreservation protocols: the classics and the new trends

New embryo cryopreservation devices

Slow freezing is commonly performed using the traditional 0.25 ml straws. However, the vitrification containers have been considerably developed throughout the last years, and new devices have been developed (Reviewed in Arav, 2014) that minimize the volume in which embryos are vitrified (0.1-1 µl). These vitrification containers, which increase the cooling and warming rates (up to 20000ºC/min), have been classified into groups: 1) surface techniques and open systems, which permit the highest cooling rate and also high warming rates by direct exposure to solutions; and 2) close systems, which allow high cooling rates with the advantage of being safer and easier to handle. The main limitation of these devices is the expensive cost and the low number of embryos that they can hold, which is a major drawback for the routinely embryo cryopreservation in polytocous animals such as pigs. To overcome this problem, systems that allow the cryopreservation of large number of embryos such as the hollow fiber (Matsunari *et al*., 2012) or the easily available method of paper container (Kim *et al*., 2012) have come up lately. Furthermore, new trends aim to automate sample preparation for mammalian embryo vitrification using digital microfluidic devices (Pyne *et al*., 2014).

Cryopreservation media and cryoprotectants

Freezing and vitrification media are aqueous cryoprotectants solutions based in either phosphate-buffered or Hepes-buffered culture media. Although some cryopreservation and warming media are commercially available for cattle, equine and pigs, most of the reported data regarding embryo cryopreservation in livestock animals have been obtained using “handmade” solutions. Commonly these media contain serum or serum components. Nowadays, there is a growing concern regarding the convenience of using chemically defined media for embryo cryopreservation, which will eliminate sanitary risk and will reduce sources of variation among laboratories. In this sense, synthetic media have been already described for bovine (Hasler, 2010) and pig embryo vitrification (Sánchez-Osorio *et al*., 2010). The commercialization of these synthetic media will be of great benefit for the widespread application of this technology.

Slow freezing and vitrification require the exposure of embryos to CPAs in order to prevent the formation of ice crystal. During the last decades a wide range of cryoprotectants has been used in embryo cryopreservation protocols, which can be divided in three groups: 1) Low molecular weight permeating CPAs such as glycerol, Dymethylsulfoxide (DMSO),

propylene glycol, ethylene glycol (EG) and other alcohols; 2) Low molecular weight non-permeating CPAs which include sucrose, trehalose and other sugars; and, 3) High molecular weight non-permeating CPAs such as polyvinyl alcohol and other polymers (reviewed in Palasz and Mapletoft, 1996).

In practice, slow freezing protocols commonly implicate the use of a single permeating CPA, whereas vitrification involves the use of mixtures of two permeating CPAs in combination with a sugar (reviewed by Palasz and Mapletoft, 1996; Kasai and Mukaida, 2004). Because vitrification requires high CPAs concentrations, toxicity is an important issue to consider when improving vitrification protocols. Such high CPA concentration can lead to chemical toxicity and osmotic injury. In this way, combinations of two permeable CPAs have been successfully used to reduce the toxicity of individual agents during the vitrification of embryos of several mammalian species (Ishimori *et al*., 1992; Vicente *et al*., 1994). Furthermore, the addition of a non-permeating CPA such as sugar or a macromolecule to the vitrification solution significantly reduces the amount of permeating CPA required for vitrification and therefore decreases the toxicity (Liebermann *et al*., 2002, Liu *et al*., 2012). The most common and accepted CPA for vitrification is EG, which has low toxicity and is highly permeable (Emiliani *et al*., 2000). Each cryoprotectant has a different permeability and thus, a balance between concentration of cryoprotectant, exposure time and working temperature needs to be determined to allow successful vitrification. Adjustments to the protocols depending on the target species or the embryonic developmental stage are also required. Thus, while DMSO, EG and sucrose is the preferred mixture for pig and bovine embryo cryopreservation, glycerol and EG seems to be the usual cryoprotectants chosen for equine embryos (Barfield *et al*., 2009; Kingma *et al*., 2011).

Slow freezing is worldwide used for *in vivo*-derived bovine, ovine and goat embryos, resulting in appropriate farrowing rates (Table 1). Vitrification has not replaced slow freezing so far, but is an alternative for situations where traditional cryopreservation procedures yield unsatisfactory results. This is the case of porcine embryos, early developmental stages and *in vitro* produced embryos (reviewed in Vajta, 2000).

Table 1. Summary of farrowing rates published after transfer of cryopreserved in vivo-derived embryos in different livestock species.

Species	Reference	Cryopreservation procedure	ET Method	Number of ETs	Number of Embryos/ recipient	Farrowing rate (%)
Bovine	Reviewed in Hasler, 2014	Slow freezing	Surgical	586	1-2	71
		Slow freezing	Non-surgical	72		60
	Van Wagtendonk-de Leeuw,1997	Vitrification	Non-surgical	393	1	44.5
		Slow freezing		335		45.1
Pig	Martinez *et al*., 2015	SOPS-Vitrification	Non-surgical	33	40	72.7
	Gomis *et al*., 2012	SOPS-Vitrification	Non-surgical	10	35	50
	Cuello *et al*., 2005	OPS-Vitrification	Non-surgical	21	20	42.9
Sheep	Bettencourt *et al*., 2009	OPS-Vitrification	Laparoscopy	11	2	54.6
		Slow freezing		19	2	68.4
	Green *et al*., 2009	OPS-vitrification	Laparoscopy	44	1	55.8
		Slow-freezing	Laparoscopy	43	1	38.6
	Papadopoulos *et al*., 2002	OPS-Vitrification	Surgical	10	2	50
	Baril *et al*., 2001	0.25 ml straws-Vitrification	Surgical	25	2	72
Goat	Guignot *et al*., 2006	Slow freezing	Surgical	26	2	69
		0.25 ml straws-Vitrification		29	2	48
		OPS-Vitrification		37	2	22
Equine	Hinrichs, 2012	DM Vitrification method	Non-surgical	8	1	75**
	Choi *et al*., 2011	DM Vitrification method	Non-surgical	7	1	71**
	Eldridge-Panuska *et al*., 2005	0.25 ml straws-Vitrification	Non-surgical	26	1	62*

ET: Embryo transfer; *Day 20 of pregnancy approximately; **Heart beat stage. OPS: Open Pulled Straw; SOPS: Superfine OPS; DM: Fine-diameter microloader pipette tips using dimethylsulfoxide.

Thawing and warming protocols

Unlike slow freezing and vitrification procedures, thawing and warming protocols are very similar, using both of them very high heat rates. Typically, embryo containers are removed from liquid nitrogen and embryos are placed in solutions with decreasing concentrations of sucrose (or other non-permeating sugar) that make the CPAs to exit the embryos by producing an osmotic gradient. Today, the development of efficient direct thawing and warming methods that allow the direct transfer of embryos without removing of CPAs is essential for the use of cryopreserved embryos under field conditions. Direct transfer protocols were first described for bovine *in vivo* derived frozen embryos (Leibo, 1984). Since then, these procedures have been widely adopted allowing the direct transfer of bovine vitrified/thawed embryos with pregnancy rates similar to the ones obtained after the transfer of fresh embryos (60%, 3/5; Saha *et al*., 1996). Currently, the main challenge in this species is to develop a direct transfer method for *in vitro* produced bovine embryos. Inaba *at al*., reported acceptable pregnancy rates with *in vitro* derived vitrified bovine embryos (44.4%, 4/9; Inaba *et al*., 2011). Although no data on calving rate have been reported from these researchers, the results seem promising. For other species, direct warming and transfer methods have also been developed for vitrified embryos with encouraging farrowing rates in pig (42.9%, 9/21; Cuello *et al*., 2005; and 50%, 5/10; Gomis *et al*., 2012), goat (56%, 14/25; Guignot *et al*., 2006) and sheep (57.1%, 12/21; Green *et al*., 2009). In addition, promising pregnancy rates have been achieved with vitrified equine embryos (62%, 16/26; Eldridge-Panuska *et al*., 2005).

Effect of cryopreservation on the embryo: what we see and what we don't see

Alterations at the cellular level

During cryopreservation there is a risk of fracture damage, which has a higher incidence in the slow freezing procedures (Kasai *et al*., 1996). In addition, the equilibrium step of any cryopreservation protocol can cause an osmotic shock that may result in a shrunken embryo. Osmotic injury can disrupt the cytoskeleton (Dobrinsky *et al*., 2000). Depolimerization of microtubules and microfilaments have been observed after cryopreservation and traditional embryo vitrification using 0.25 ml straws (reviewed in Dobrinsky, 1997). To avoid this damage, cytoskeletal stabilizer agents such as Cytochalasin B has been proposed during the vitrification process in pigs. However, when ultra-rapid vitrification procedures were used to cryopreserved porcine morulae and blastocysts, cytoskeletal stabilizers were not necessary (Tharasanit *et al*., 2005; Cuello *et al*., 2010).

Slow freezing (Fair *et al*., 2001; Dalcin *et al*., 2013) and vitrification (Fabian *et al*., 2005; Cuello *et al*., 2007a; Dalcin *et al*., 2013; Chrenek *et al*., 2014) cause ultrastructural changes in embryos such as accumulation of cellular debris, an increase of vesicles and changes in the trophoblastic microvilli. Cryopreservation also induces abnormal distribution of mitochondria (Nagai *et al*., 2006), mitochondria swelling, alteration in the mitochondria shape and the rupture of their membranes (Cuello *et al*., 2007a). Recently, Dalcin *et al*. (2013) reported not only morphological alterations but also disturbed mitochondrial activity in frozen and vitrified embryos. Since mitochondria are essential for aerobic metabolism and ATP production in the embryo, the addition of glycine as a protector of mitochondria to the vitrification media has been proposed in order to overcome the above mentioned detrimental effects (Zander-Fox *et al*., 2013).

Despite all these morphological changes at a cellular level, if they are slight, the embryo is able to regenerate and eliminate death cells. In this case, the normal morphology of the embryo can be almost entirely restored after 24 h of culture without affecting the embryo viability (Vajta *et al*., 1997).

Alterations at the molecular level

Slow freezing and vitrification affect the DNA integrity (Cuello *et al*., 2005, Fabian *et al*., 2005, Kader *et al*., 2009). Considering that increased DNA fragmentation in cryopreserved embryos is partly caused by a surplus of free radicals, the addition of antioxidants to media could reduce this effect (Hosseini *et al*., 2009). Cryopreserved embryos have also shown altered expression of a number of genes when compared to fresh embryos (Mamo *et al*., 2006; Stinshoff *et al*., 2011; Shaw *et al*., 2012). Most of these alterations are related to homeostasis, metabolism and regulation of cellular and physiological activities such as cell proliferation, the cell cycle, developmental, biosynthesis, respiration and stress-related gene expression (Boonkusol *et al*., 2006; Mamo *et al*., 2006; Stokes *et al*., 2007). For example, altered Heat shock protein A1 and Solute Carrier 2 A3 gene expression has been observed in frozen-thawed embryos, which are indicators of heat stress and solute carrier functions (Kuzmany *et al*., 2011; Stinshoff *et al*., 2011). It seems logical that embryos tried to compensate for the osmotic shock and cold-conditions by altering their metabolism. This adaptation and plasticity has consequences for the embryo and little is known about the potentially effect on them and the subtle effect on the offspring (Thompson *et al*., 2007). On the other hand, the endometrium, considered as ultimate sensor of quality and healthy state of the embryos, may distinguish between fresh and vitrified embryos (Almiñana *et al*., 2014), as demonstrated by the altered gene expression

of the uterus towards the frozen embryos when compared to fresh embryos. Moreover, in rabbits it has been observed that vitrification modifies the pattern of gene and proteins expression in the placenta after implantation (Saenz-de-Juanjo *et al*., 2014).

Furthermore, when metabolism alterations were monitored in regard of the pyruvate uptake in vitrified and frozen embryos, slow-frozen embryos were more metabolically impaired than those that were vitrified (Lane *et al*., 2002). Research so far implies that vitrification induces less negative alterations on the embryo proteome and energy metabolism than slow freezing (Varghese *et al*., 2009).

Taken together, the altered gene, protein and metabolic expression of cryopreserved embryos with the differently response of the endometrium to frozen and fresh embryos may explain the inferior farrowing rates obtained with cryopreserved embryos compared to fresh ones (Papadopoulos *et al*., 2002; reviewed in Hasler *et al*., 2014).

Epigenetic effects

There is an increasing concern that cryopreservation may induce epigenetic marks and long-term alterations in the embryo. To date, there are very limited and contradictory studies regarding the possible epigenetic effects of the cryopreservation process on the embryo. On one side, some research has shown that vitrification does not alter gene methylation patters in mouse blastocyst (Zhao *et al*., 2012). On the other side, vitrification has been found to increase gene methylation in bovine two-cell embryos (Zhao *et al*., 2012). Moreover, the process of embryo vitrification itself significantly augmented the loss of methylation in the *H19* differentially methylated domain in mouse foetuses derived from vitrified embryos (Wang *et al*., 2010). DNA methylation is a key epigenetic modification, which is essential for normal embryonic development. The complex DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs). Recently, Petrussa *et al*. (2014) reported that cryopreservation resulted in disturbed expression patterns of DNMTs in human preimplantation embryos. These findings call up for further research to assess whether these disturbed embryonic DNMT expression patterns may have long-term developmental consequences for the embryo.

Personal considerations

The aim of this point is to go through personal considerations and practical aspects to take into account when embryo cryopreservation is used in farm animals.

How are embryos selected for cryopreservation?

The greatest factor affecting freezability is embryo quality, a feature that is difficult to evaluate objectively. To date, morphology evaluation by stereomicroscopy is the most employed and useful tool to evaluate embryo quality (Cuello *et al*., 2007a; Dalcin *et al*., 2013). Currently, time-lapse imaging of preimplantation embryos has been suggested as a helpful tool that may allow embryologists to be more objective in scoring embryos. Time-lapse data in conjunction with traditional morphology embryo scoring may allow better selection of embryos for cryopreservation and subsequent transfer (Conaghan *et al*., 2013).

For how long can an embryo be cryopreserved?

It has been demonstrated in different species that long storage of frozen embryos has no effect on their post-thaw survival, implantation rates, clinical pregnancy, miscarriage and live birth (up to 20 years in humans: Riggs *et al*., 2010; up to 3 years in pigs: Sanchez-Osorio *et al*., 2010; after 15 years in bovine: Fang *et al*., 2014; after 7.5 years in Sheep: Yao *et al*., 2012). Storage of cryopreserved embryos for long-term periods requires temperatures below -130ºC, the glass transition temperature of water, which can be easily provided by storage in liquid nitrogen containers at -196ºC. In fact, Glenister *et al*. (1984) pointed out by using a mouse experimental model, that frozen embryos stored in liquid nitrogen will remain “alive” for at least 2000 years. In the light of these results, it seems that the major limitation for the long-term embryo storage will be the cost for the high amount of liquid nitrogen required and the storage space and equipment associate to liquid nitrogen demands. The current development of breakthrough technology that allows the storage of cells and gametes in dry state could overcome this inconvenient in a future (Arav, 2014).

Does size matter? When being big is a problem and being small is crucial

Size really matters when it comes to embryo cryopreservation. Cryopreservation of equine embryos represents a challenge related to their size (reviewed in Stout, 2012). While the use of slow freezing and vitrification methods in small equine blastocysts (<300 µm of diameter) has been effective, their use in expanded blastocysts (>300 µm of diameter) has resulted in poor outcomes after transfer (Hinrichs, 2012). Timing of the period to obtain small equine blastocysts is uncertain and it is limited to 24 h (Betteridge, 2007). This technical difficulty makes necessary to improve the cryopreservation of expanded blastocysts. The problems associated to the cryopreservation of expanded equine blastocysts seem to be related to the blastocoele size and to the presence of the embryonic capsule (reviewed in Stout, 2012; Hinrichs, 2012). Currently, the reduction of equine

embryo size by aspiration of blastocoel content with a piezo drill or laser has been proposed (Choi *et al*., 2011; Scherzer *et al*., 2011). These studies have reported promising results on embryo survival rates, suggesting that the large volume of equine embryos is the primary impediment for successful cryopreservation.

But not only the size of equine embryos is a challenge, the size of the vitrification drop is also a matter of concern. A minimal volume is one of the most important factors for the effectiveness of vitrification as mentioned above (reviewed in Arav, 2014). Thus, keeping the volume as small as possible is a "must" when using any vitrification device.

Does fat matter? The lipid composition of the embryo

In this case, the matter of concern is more related to the impact of the fat in the cryopreserved embryo. The large amount of lipid droplets in the embryo cytoplasm, which is more evident in some species like pigs, in *in vitro* produced embryos and in early developmental stages, makes the embryos more sensitive to chilling injuries. To overcome this problem, mechanical removal of lipids from the embryo prior cryopreservation was proposed in pigs (Nagashima *et al*., 1995). However, since manipulations that disrupt the zona pellucida should be avoided, partial delipidation by chemical agents has been proposed as a more adequate strategy. In this sense, Forskolin (Men *et al*., 2006; Cuello *et al*., 2013) and L-carnitine (Takahashi *et al*., 2013) have proved to increase the cryopreservation ability of early and *in vitro*-derived embryos.

Cryopreservation "à la carte"

As we have been mentioning, the efficiency of any cryopreservation protocol is affected mainly by the species, the embryo quality, the origin of the embryo (*in vivo*- or *in vitro*-derived) and the developmental stage. Each species is unique in many aspects and thus, what may work for one species, might not work for another. While satisfactory results have been obtained after transfer of cryopreserved embryos in human, mice, cattle, pig, sheep and goat, poor results have been achieved in other domestic species such as equine (Table 1). Differences in the cryopreservation ability among species have been partly ascribed to differences in the embryo lipid composition. This is the reason for the low success of slow freezing in pigs (Polge and Willadsen, 1978; Nagashima *et al*., 1994). Moreover, embryos from different species present special features that may affect the cryopreservation procedures, such as the equine embryonic capsule (Hinrichs, 2012) or the different permeability to the CPAs (Jin *et al*., 2011).

Embryo size, water, lipid content and permeability of the plasma membrane vary not only among species but also among different developmental stages in the same species, which affect the cryopreservation outcome (Agca *et al*., 1998; Sánchez-Osorio *et al*., 2008; Jin *et al*., 2011). Although most embryo cryopreservation studies in human have shown slight or no differences in post-thaw survival among embryos frozen at the pronuclear, cleavage or blastocyst stage (Salumets *et al*., 2003; Moragianni *et al*., 2010), pig studies have demonstrated that morula and blastocyst are superior for vitrification purposes than 2-4 cells embryos based on embryo survival rates (Cuello *et al*., 2007b). In the same line, Asgari *et al*., (2012) and colleagues observed that the potential of 5-8 cell stage bovine embryos to survive vitrification and develop to the blastocyst stage was significantly lower than vitrified 8-16 cells and morula stage embryos. Differences in the cryopreservation ability have been also observed depending of the embryo origin (*in vivo vs. in vitro*). The high sensitivity to chilling injury and freezing of *in vitro*-derived embryos has been associated to their higher lipid content (Romek *et al*., 2009) and their lower quality (reviewed in Rizos *et al*., 2008) compared to *in vivo*-derived embryos.

To date, the perfect embryo cryopreservation protocol has not yet been established. A preliminary screening for each species of interest and embryo characteristic (developmental stage, origin, etc) is required to select the optimal cryopreservation conditions. Thus, we propose that a cryopreservation protocol "*à la carte*" may lead us to the best results for each occasion.

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Developmental programming in the preimplantation period: can it be exploited to enhance postnatal function in cattle?

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Abstract

The concept of developmental programming states that the function of an adult animal depends on environmental conditions to which it was exposed to before birth. Developmental programming can occur in the preimplantation period. Accordingly, certain environmental signals, acting either on the mother (for pregnancies established *in vivo*) or on the embryo directly (for cultured embryos), can program development of the preimplantation embryo to have effects on postnatal life. It is proposed that research on developmental programming in cattle could lead not only to elimination of adverse outcomes associated with *in vitro* production of embryos but also to discovery of approaches to produce a neonatal animal with superior prospects for achieving optimal production later in life.

Keywords: developmental programming, environment, livestock production, preimplantation embryo.

Introduction

Programming of the preimplantation embryo – A seldom-travelled frontier for the animal scientist

It has long been the goal of animal scientists to devise means to improve the efficiency at which agriculturally-important animals produce products useful for man. Most commonly, enhanced production has been achieved through a combination of selection of genes that are optimal for production and provision of an environment that maximizes the opportunity for those genes to be expressed. Thus, for example, the increase in milk yield in dairy cattle breeds such as the Holstein and Jersey has depended upon intensive and accurate selection for genes conferring females with the capacity to produce large amounts of milk as well as provision of cattle with nutrients, housing and other environmental conditions that optimize milk yield.

The function of an adult animal depends not only on the environment to which it was exposed after birth but also on environmental conditions it was exposed to before birth. This concept, which has been variously termed developmental programming, fetal programming or developmental origins of health and disease, has been documented not only in mammals (Roseboom *et al*., 2001; Ganu *et al*., 2012; Walker and Ho, 2012; Fleming *et al*., 2015), but also amphibians (Berg *et al*., 2009), reptiles (Schwanz*et al*., 2013) and fish (Meier *et al*., 2010; Celeghin *et al*., 2011). For mammals, the environment of the conceptus is established by its mother and changes in maternal environment can alter postnatal phenotype of the developing organism.

Postnatal function can be programmed by alterations in maternal environment throughout the length of gestation, including as early as the preimplantation period (Kwong *et al*., 2000; Calle *et al*., 2012) and as late as the final stages of gestation (Li *et al*., 2013; Tao *et al*., 2014; Master *et al*., 2015). Indeed, there is experimental evidence that alterations in parental function affecting male gametes (Lane *et al*., 2014; Master *et al*., 2015) can influence postnatal phenotype of the offspring.

Those who work with the *in vitro* produced (IVP) embryo are well aware that an inadequate environment during the preimplantation period can adversely change the developmental outcome of the embryo. Some of the specific consequences of exposure of the embryo to an inadequate culture environment will be detailed in a subsequent section. Not all programming events during the preimplantation period need be harmful, however. Although the evidence is fragmentary, it is hypothesized that certain environmental signals, acting either on the mother (for pregnancies established *in vivo*) or on the embryo directly (for pregnancies established *in vitro*), can program development of the preimplantation embryo to have beneficial effects on postnatal life. The purpose of this paper is to encourage research directed towards this hypothesis because, if true, both the animal manager and the embryologist will have new tools with which to optimize animal production.

Lessons from the mouse regarding developmental programming in the preimplantation period

The mouse has been the preeminent species used to document the idea that changes in the maternal environment during the preimplantation period can alter development of the embryo in a way that alters postnatal phenotype. The best studied example is for offspring born to mothers that were fed a low protein diet during the first 3.5 days of gestation (i.e., through the period of blastocyst formation). An example of representative results is shown in Fig. 1. As for many other cases of developmental programming, consequences of being gestated in a mother fed a low protein diet during the

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preimplantation period depend on the sex of the embryo. Female offspring of females fed a low protein diet during the first 3.5 days of gestation experienced increased body weight (Watkins*et al*., 2008, 2011), decreased ratio of heart weight to body weight (Watkins *et al*., 2008), higher expression of *Insr* and *Igf1r* in retroperitoneal fat (Watkins *et al*., 2011), and altered behavioral responses (Watkins *et al*., 2008). Male offspring displayed attenuated vasodilation response in mesenteric arteries (Watkins *et al*., 2010). For both sexes, being derived from mothers fed a low protein diet during the preimplantation period resulted in increased systolic blood pressure (Watkins *et al*., 2008, 2011), elevated amounts of angiotensin converting enzyme in the lung (Watkins *et al*., 2010), and lower expression of *Ucp1* in retroperitoneal fat (Watkins *et al*., 2011).

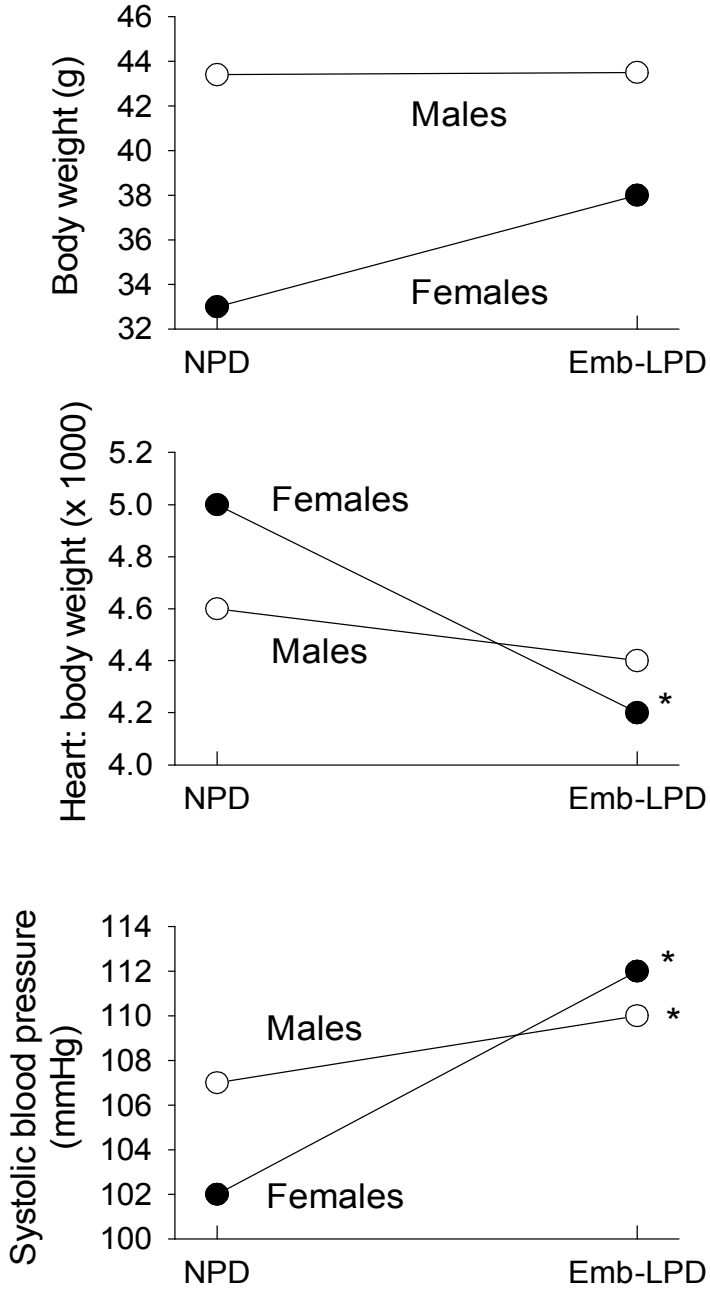

Figure 1. Developmental programming in the mouse caused by maternal protein nutrition in the preimplantation period. Female mice were fed a diet containing 18% casein throughout gestation (normal protein diet; NPD) or were fed a diet containing 9% casein from mating to day 3.5 of gestation and then fed an 18% casein diet thereafter (embryo low protein diet; Emb-LPD). Shown in the figure are data on the male (open circles) and female (closed circles) offspring at 21 days of life. Asterisks represent significant effects of Emb-LPD ($P < 0.05$). Note that, while effects on body weight at 21 days were not significant, Emb-LPD females were significantly heavier than NPD females at other times examined (results not shown). Data are from Watkins *et al*. (2008).

Other maternal events during the preimplantation period that have been reported to affect postnatal phenotype in mice are lipopolysaccharide (LPS) challenge (Williams *et al*., 2011) and mating of female to males in which seminal vesicles were removed surgically (Bromfield *et al*., 2014). For these stimuli, also, alterations in postnatal function depended on sex. For example, males born as a result of mating females to vesiculoectomized males were fatter than control males whereas there was no effect on adiposity for females (Bromfield *et al*., 2014).

It is possible that maternal events during the preimplantation period do not reflect alterations of embryonic function directly but rather changes in maternal physiology that effect the conceptus later in pregnancy. This is not the case for low protein feeding or mating to vesiculoectimized males because alterations in postnatal phenotype remain when embryos are recovered from affected females and transferred to control recipients (Watkins *et al*., 2008; Bromfield *et al*., 2014). At least some of the alteration in conceptus development caused by low protein feeding and mating to vesiculoectomized males is probably caused by altered placental function because both treatments alter placental growth (Bromfield *et al*., 2014; Watkins *et al*., 2015) and, for low protein feeding, function of trophectoderm (Sun *et al*., 2014) and primitive endoderm (Watkins *et al*., 2008; Sun *et al*., 2015). Accompanying alterations in placental function are changes in gene expression and DNA methylation of *Gata6* in primitive-endoderm-like embryoid bodies derived from embryos harvested from mothers fed low protein diets (Sun *et al*., 2015).

Taken together then, the lesson from the mouse is that changes in maternal environment during the preimplantation period can alter subsequent development in a sex-dependent manner that results in a change in phenotype in adult life.

The *in vitro* produced embryo – A sometimes victim of developmental programming

There are many alterations in embryonic function associated with IVP. These include changes in morphology (Crosier *et al*., 2001; Rizos *et al*., 2002a), gene expression (Driver *et al*., 2012), cryotolerance (Enright *et al*., 2000; Al-Katanani *et al*., 2002), and competence to establish pregnancy after transfer to females (Pontes *et al*., 2009; Siqueira *et al*., 2009). At least some of these defects are caused by the culture environment during the preimplantation period rather than to problems derived from the oocyte or induced in the fertilization process. Transfer of IVP embryos to the sheep oviduct improved survival to cryopreservation (Rizos *et al*., 2002c) and made gene expression more similar to embryos produced *in vivo* (Rizos *et al*., 2002b).

The IVP bovine embryo can also experience alterations in development later in pregnancy as indicated by occurrence of fetal growth abnormalities (Farin *et al*., 2006), loss of imprinting (Chen *et al*., 2015), and increased neonatal mortality (Bonilla *et al*., 2014). Clearly, then, the *in vitro* produced embryo can experience abnormal developmental programming.

What is not clear in the bovine is whether consequences of IVP extend into the postnatal period. The one study to examine this question, using small numbers of animals, did not find any difference between calves produced by IVP with calves produced by artificial insemination in terms of calf growth, age at first service, percent of heifers pregnant at first service, or milk yield or composition in the first 120 days of the first lactation (Bonilla *et al*., 2014). However, results from embryos produced *in vitro* in the mouse and human are indicative that the question should be examined more closely. Thus, in the mouse, alterations in culture conditions can cause sex-dependent changes in adult phenotype (Fernández-Gonzalez *et al*., 2004; Sjöblom *et al*., 2005; Serrano *et al*., 2014). Results in the human are more difficult to interpret because of the overrepresentation of older and more infertile couples as parents of children derived from *in vitro* fertilization (IVF) as compared to children from natural conceptions. With this caveat in mind, it is important to note that there are reports from a Dutch cohort of singleton children aged 8-18 that derivation by IVF was associated with increased body fatness (Ceelen *et al*., 2007), systolic and diastolic blood pressure (Ceelen *et al*., 2008), and fasting concentrations of glucose (Ceelen *et al*., 2008). In another study of Swiss singleton children, Scherrer *et al*. (2012) found evidence of vascular dysfunction for children born following IVF or intracytoplasmic sperm injection (average age 11) as compared to those born following natural conception (average age 13). There were no differences in body weight or fatness, circulating concentrations of lipid, glucose or insulin, glucose tolerance or glucose resistance (Scherrer *et al*., 2012).

Unfortunately, too few experiments with IVP embryos in cattle monitor pregnancies to term or ascertain the function of the resultant offspring during later life. This oversight should be corrected whenever feasible because it is possible that there are negative consequences of IVP on postnatal health, growth, reproduction or lactation. It might even be that there are specific culture conditions used for IVP that have beneficial effects on specific physiological functions important for optimal production.

Alterations in maternal environment during the periconceptional period can change adult phenotype in ruminants – lessons from the sheep

The fact that fetal development in the cow can be disrupted following IVP (Farin *et al*., 2006; Bonilla *et al*., 2014; Chen *et al*., 2015) is indicative that preimplantation developmental programming can occur in cattle. To date, however, there are no reports from cattle as to whether alteration of maternal environment during the preimplantation period can also modify postnatal characteristics of the offspring. Results from another ruminant, the sheep, would indicate that such a phenomenon can occur.

The first such study in sheep indicating the importance of the periconceptional environment of the mother for characteristics of the offspring in adulthood utilized a model in which ewes serving as embryo donors were fed a diet deficient in cobalt and sulfur designed to reduce capacity for DNA methylation (Sinclair *et al*., 2007). The experimental diet was fed from 8 weeks before breeding until 6 days afterwards. Embryos were then flushed from the uterus and transferred to control females to ensure effect of maternal diet reflected actions on the embryo. At day 90 of gestation, there were diet-associated differences in methylation status of 4% of 1000 CpG islands examined in fetal liver. Also, animals that were derived from females fed the experimental diet had several altered physiological characteristics in adulthood. For both sexes, body weight was greater in the treated group. For males only, offspring from mothers fed the experimental diet were fatter, had greater haptoglobin response to ovalbumin immunization, increased insulin resistance and elevated diastolic blood pressure.

The design of the experiment by Sinclair *et al*. (2007) did not make it possible to determine whether actions of maternal feeding were on the embryo itself or the oocyte from which it was derived. However, the potential for manipulating postnatal function of animals by modifying maternal diet in the periconceptional period was made clear. Moreover, there are other reports where an alteration in maternal environment either in the preovulatory period and early pregnancy or in early pregnancy alone changed postnatal outcomes in offspring. Results are summarized in Table 1. Changes in maternal environment reported to cause changes in postnatal phenotype of the offspring are undernutrition (Gardner *et al*., 2004, 2006; Poore *et al*., 2007, Hernandez *et al*., 2010) and injection with sustained release growth hormone (GH) ~3 days before estrus (Costine *et al*., 2005; Koch *et al*., 2010). The phenotype caused by these manipulations of the maternal system are less profound than for the study of Sinclair *et al*. (2007), probably because the strategy of limiting DNA methylation employed in the latter study caused a larger change in the fetal epigenome than that caused by undernutrition or activation of the GH-IGF1 axis.

Colony Stimulating Factor 2 – A maternal signal that modifies the developmental program of the preimplantation embryo

Actions of the mother to alter the developmental program of the preimplantation embryo are likely mediated in part by embryo regulatory molecules produced by the oviduct or endometrium. These molecules, which in the cow include activin, CSF2, DKK1, EGF, FGF2, IGF1, IL1B, LIF and TGFB (Hansen *et al*., 2014a), have been termed embryokines because of their capacity to regulate embryo growth and differentiation (Hansen *et al*., 2014a, b). One of these molecules, CSF2, can exert actions on the preimplantation embryo that alter development later in gestation and in postnatal life.

The first indications that CSF2 can regulate the developmental program of the preimplantation embryo was the finding of Sjöblom *et al*. (2005) in the mouse that addition of CSF2 to culture medium prevents the otherwise deleterious effects of embryo culture on postnatal phenotype. Addition of CSF2 to culture medium from the two-cell to blastocyst stages reduced or prevented effects of culture on postnatal growth in females and males, relative brain mass in males and placental weight of female progeny when they themselves became pregnant. There was no alleviation of the effect of culture on fatness of males.

Treatment of bovine embryos from day 5 to 7 of development with CSF2 also affects later development in a manner that varies between female and male embryos (Dobbs *et al*., 2014). After culture, embryos were transferred to cows and flushed from the reproductive tract 8 days later, at day 15 of gestation. Day 15 was chosen because the bovine embryo is undergoing rapid elongation of the trophoblast and secretion of the antiluteolytic molecule IFNT (Spencer *et al*., 2007). There was aninteraction between sex and treatment for conceptus length and concentrations of IFNT in the uterine lumen (an indirect measurement of embryonic production of IFNT). CSF2 decreased embryo length and intrauterine accumulation of IFNT in females but increased length and IFNT in males (Fig. 2). In addition, effects of IFNT on gene expression and DNA methylation in the trophoblast also varied between female and male embryos.

Table 1.Changes in postnatal phenotype in sheep caused by alterations in maternal environment during the preovulatory period and/or early pregnancy.

Maternal treatment	Age examined	Altered postnatal phenotype			Notes	Reference
		Both sexes	Females only	Males only		
Restricted feed intake from day 1-30 of gestation	1 year	• increased pulse pressure • reduced rate pressure product • lack of tachycardia after angiotensin II infusion • reduced baroreflex sensitivity during angiotensin II infusion			Sex differences not determined	Gardner *et al*., 2004
Restricted feed intake from day 1-30 of gestation	1 year		• increased resting cortisol • reduced ACTH and cortisol response to CRH and vasopressin	• increased ACTH and cortisol response to CRH and vasopressin		Gardner *et al*., 2006
Restricted feed intake, day 1-31 of gestation	1.5 and 2.5 years			• increased insulin resistance (1.5 years only)	most effects not significant	Poore *et al*., 2007
Reduced feed intake, day -2 to 30 after mating	4 months			• increased cortisol response to isolation • increased escape attempts, behavioral test		Hernandez *et al*., 2010
Sustained release GH, ~ day -3 before breeding	30-75 days		• increased body weight	not examined	only females examined	Costine *et al*., 2005
Sustained release GH, ~ day -3 before breeding	100 days		• increased body weight • Decreased IGF1 response to GHRH challenge	not examined	only females examined	Koch *et al*., 2010

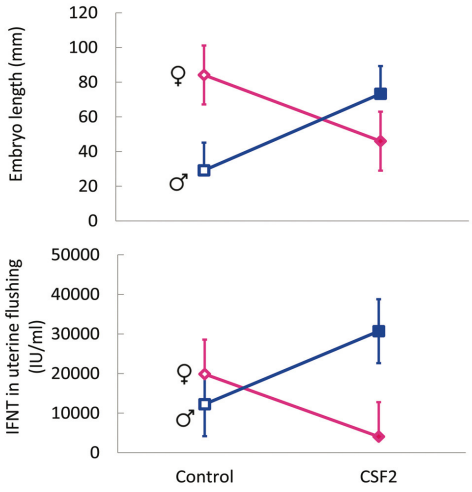

Figure 2.Sex of embryo affects actions of CSF2 treatment from day 5-7 of development on conceptus length and concentration of IFNT in uterine flushing at day 15 of pregnancy. The figure is reproduced from Biology of Reproduction(Dobbs *et al*., 2014).

Where do we go from here?

The focus of this paper has been on the preimplantation period. This is not the only time in pregnancy when alterations in maternal environment can change the characteristics of development to affect postnatal function of the offspring. In cattle, for example, diet in the second or third trimester can affect fertility (Martin *et al*., 2007) and carcass characteristics (Micke *et al*., 2010a, b, 2011) of the offspring. The fact that maternal events can affect the developmental program of the conceptus means that opportunity exists for shaping postnatal function of livestock through manipulation of maternal environment during gestation. Even though there has long been interest in exploring prospects for regulating offspring characteristics of livestock species by manipulating events during pregnancy (see Everett, 1964 for one early example), the field remains small in relation to the potential for gain in animal productivity. More should be done.

As compared to other times in pregnancy, the preimplantation period offers unique advantages. Not only is it possible to modify maternal function during this period of pregnancy but, in cases of IVP, the embryo spends part of the preimplantation period outside the mother. Experiments with CSF2 (Sjöblom *et al*.,2005; Dobbs *et al*., 2014)show that it is possible to alter the environment of the cultured embryo to change the trajectory of development. Unfortunately, exposure of the embryo to culture conditions often causes the developmental program to become dysregulated (Farin *et al*., 2006). With more research focused on outcomes of IVP beyond the establishment of pregnancy or even calving, it may be possible to not only eliminate adverse outcomes associated with IVP but to also produce a neonatal animal with superior prospects for achieving optimal production later in life.

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Metaboloepigenetics: providing alternate hypotheses for regulation of gene expression in the early embryo

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Abstract

The metabolic and epigenetic landscapes of the pre-implantation embryo change and evolve rapidly as the embryo travels through the reproductive tract. The maternal and paternal genomes combine, rapid cell division is initiated, potency is re-established and eventually differentiation begins, all in the absence of a vascular supply delivering oxygen, nutrients and a functional waste removal system. In recent years, it has become clear that environmental challenges to the developing embryo, including maternal diet, stress and inflammation, alter its long-term trajectory, although the exact signaling molecules, which are recognised by the embryo, and the mechanisms by which these signals are translated into long-term outcomes, remain elusive. Recently, it has become apparent that energy or fuel-sensing metabolic pathways interact with important epigenetic regulators of chromatin structure, to regulate gene expression. While this has not yet been explored in the pre-implantation embryo, the interaction between these two key cellular systems, - metaboloepigenetics - is a plausible mechanism by which gene-environment interactions occur, and by which the embryo's trajectory is established. This review explores the metabolic and epigenetic plasticity of the early embryo, and how the two systems intertwine to propagate the next generation.

Keywords: embryo, epigenetics, metaboloepigenetics, metabolism, transcription.

Metabolism: powering pre-implantation embryo development

Metabolism in the pre-implantation embryo is the co-ordination of energy intake, production and use, which allows the embryo to sustain the rapid cell division in a highly unique a vascular environment, required for propagation of the species. In animal cells, mitochondria are the organelles, which have evolved to most effectively produce energy, and their activity has been heavily studied in pre-implantation embryos in many species. The embryo's requirements for energy substrates change rapidly as development proceeds, favoring oxidative phosphorylation in the early stages, and glycolysis closer to implantation.

The ability for the embryo to undergo pronuclear formation, syngamy, embryonic genome activation, successive mitoses, compaction, lineage differentiation and blastocoel development are based on the intrinsic capacity of the embryo to regulate the temporal and spatial distribution and consumption of energy. Decades of elegant work in several species have explored the energetic requirements of the pre-implantation embryo, both *in vivo*, and under varied *in vitro* conditions. The early cleavage embryo is almost entirely dependent on oxidation of substrates including pyruvate (Gardner and Leese, 1988; Butcher *et al*., 1998), lactate (Lane and Gardner, 2000) and amino acids (Gardner and Lane, 1993; Van Winkle, 2001) to sustain the production of ATP. A primary function of this pyruvate-to-lactate conversion is thought to be the regeneration of NAD^+ for subsequent use in glycolysis, which, although normally occurring under anaerobic conditions, occurs in the presence of oxygen in the reproductive tract (Krisher and Prather, 2012). During this period of mitotic cell division, the embryo has been proposed to have a "quiet" metabolism (Leese, 2002; Baumann *et al*., 2007), partially because although DNA replication and cell division are occurring, cellular volume decreases with each division (Turner *et al*., 1994), maintaining a moderate requirement for energy, and in turn, oxygen. In contrast, evidence in human embryos suggested that embryos which resulted in a clinical pregnancy had higher glucose consumption than those that did not (Gardner *et al*., 2011). Perhaps it is the fine balance between energy consumption and utilization, which determines the long term embryo's viability.

With an increased requirement for protein synthesis and transcription, and the necessity of blastocoel formation, there is an up-regulation from the "quiet" metabolic homeostasis, to a dramatically higher level, which is associated with a switch away from oxidative phosphorylation towards glycolysis (Leese, 1995), with the embryo demonstrating a significant capacity for aerobic glycolysis (Gardner and Leese, 1988). The mechanisms by which this switch occurs remain unclear, but it appears to be regulated by the presence of glucose, increasing the expression of glucose transporters (reviewed in Purcell and Moley, 2009). This dramatic increase in the metabolic capacity of the embryo is likely necessary to power the Na,K-ATPase, initially pumping fluid into the intracellular

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spaces, and later into the extracellular spaces, forming the blastocoel cavity (Biggers *et al*., 1977; Borland *et al*., 1977a, b). Ion transport systems for Na^+, Cl^-, K^+, Ca^{2+} and Mg^{2+} concentrate ions within the blastocoel, further supporting the water movement necessary for formation of the fluid-filled cavity (Borland *et al*., 1977a, b).

It has been proposed that this switch from oxidative phosphorylation to glycolysis prepares the embryo for the hypoxia it will face from the time of implantation until remodeling of the spiral arterioles of the human placenta at the end of the first trimester (Burton *et al*., 2010; Cartwright *et al*., 2010), and perhaps explaining the choice for inefficient energy production. Additionally, the glucose provides the pentose sugars for nucleic acid synthesis, and is required for both phospholipid and non-essential amino acid biosynthesis, which supports the rapid cell division (O'Fallon and Wright, 1986; Wales and Du, 1993; Cairns *et al*., 2011; Gutnisky *et al*., 2014). Although an inefficient means for generating ATP, perhaps this metabolic adaptation is important in biomass production and redox regulation, and has been likened to the Warburg Effect, a hallmark of highly proliferative cells, particularly cancer cells (Krisher and Prather, 2012).

Metabolic plasticity allows for adaptation to stress induced by *in vitro* culture, diet and other environmental challenges and contaminants. Prior to embryonic genome activation (which is as late as the 4th mitosis in some species), levels of active transcription are very low or non-existent, and metabolism provides a mechanism by which the embryo can still respond to altered surroundings. But is it possible that this metabolic plasticity, which promotes embryo survival in the absence of transcription, also has detrimental effects? Is altered metabolism changing the abundance of stored transcripts, or altering the epigenetic landscape, thereby changing the trajectory of the embryo forever? Is the aerobic conversion of pyruvate to lactate in the embryo necessary for the maintenance of NAD^+, which is known to regulate families of chromatin-modifying enzymes?

Epigenetics: a changing landscape during pre-implantation embryo development

It should come as no surprise that the epigenome of the early embryo is a rapidly changing landscape. The sperm and egg, two highly specialised cell types, come together, undergo thorough chromosomal rearrangement to fuse the maternal and paternal genetic material, and then begin to divide as a single entity, no longer highly specialised, but with the ultimate potential to form every cell type. Epigenetics, or the study of cellular traits and phenotypes that are mitotically inheritable, is the mechanism by which these highly specialised characteristics are erased, and potency re-established (Santos *et al*., 2005; Goldberg *et al*., 2007; Shi and Wu, 2009). This potency again becomes restricted, with each cell division progressively restricting the range of developmental outcomes. Although the concept sounds simple, the field of early embryo epigenetics has been wrought with inconsistency, controversy and challenge. Concurrent with technological advancement, the field of epigenetics has grown to include not only DNA methylation, the best described epigenetic modification in the pre-implantation embryo, but also histone modification and non-coding RNA, just to name a few.

Early studies describing the DNA de-methylation patterns of the early embryo suggested two distinct phases: a global, active (non-replication dependent) round of demethylation of the paternal genome, followed by a progressive, passive loss of methylation of the maternal genome as cell division proceeded (Mayer *et al*., 2000; Morgan *et al*., 2005). This active loss of paternal genome methylation was supported by studies confirming that Ten-Eleven Translocase family member, TET3, was responsible for the conversion of 5-methyl cytosine to 5-hydroxymethyl cytosine, and its subsequent removal via iterative oxidation (Gu *et al*., 2011; Wossidlo *et al*., 2011).

Most recently, single base resolution MethylC-seq revealed that during pre-implantation embryo development, most functional genomic elements undergo significant demethylation, except CpG islands (CGIs) and 5' untranslated regions (UTRs) whose methylation levels are already very low in gametes (Wang *et al*., 2014). Additionally, they demonstrated that by generating single-base resolution, allele-specific whole-genome methylomes, the paternal methylome and at least a significant proportion of maternal methylome goes through active demethylation during embryonic development, based on the presence of the oxidised methyl cytosine bases (5hmC, 5fC; Wang *et al*., 2014). With the possibilities of single cell, single base resolution methylation analysis within reach, we are likely to see increased clarity and understanding not only of the roles of DNA methylation in functional and structural genomics - including heterochromatin formation, X-chromosome inactivation and genomic imprinting - but also of epigenetic heterogeneity during early development, and the mechanisms by which loci-specific methylation alterations occur.

It is important to recognise, however, while DNA methylation is the epigenetic mechanism, which has been most extensively researched in the early embryo, many others are active and function to regulate chromatin structure, transcription and cell division. Histone modification has been explored in a number of species, at all stages of pre-implantation embryo development (reviewed in Beaujean, 2014a, b). Many modifications on H2, H3 and H4 have been described, although the technologies to date have been predominantly via immunohistochemistry (IHC), which allows for the global analysis of only one single

modification at a time. Current technologies for exploring loci-specific changes in multiple histone modifications aren't yet possible for single cell analysis, and consequently very little is known about the loci-specific changes in adjacent modifications during development. What is clear is that when the paternal genome enters the oocyte at fertilisation, the protamine is removed and replaced with oocyte-derived histones (McLay and Clarke, 2003). Although the histones are maternally-derived, the maternal and paternal DNA display dramatic asymmetry of a number of described histone modifications (Reik *et al*., 2003; van der Heijden *et al*., 2005), which is detectable until the four cell stage for some modifications.

It is clear that the most active time for epigenetic remodelling corresponds with the embryo's greatest sensitivity to the health of the maternal and paternal milieu, through the environment created within the reproductive tract, which may present both metabolic and immune challenges. The question that remains poorly answered is 'how'? Here we outline the evidence for how metabolism links in with epigenetic stability mechanisms, enabling the plasticity necessary for development, as well as long-term adaptation to the peri-conception environment.

Metaboloepigenetics: can metabolism alter the epigenetic landscape of the pre-implantation embryo?

In recent years, a number of hypotheses have emerged that propose an interaction between the epigenome and cellular metabolism which results in alterations to cell phenotype. If this is the case, stimuli or environmental changes that alter metabolism during a period when the epigenetic landscape of the embryo is naive, could alter the long-term trajectory. Epigenetics involves alterations of the DNA and chromatin by a range of enzymes which add and subtract a number of chemical modifications including methyl, acetyl and phosphate groups (Bannister and Kouzarides, 2011). The wide variation in modification types as well as the high number of potential target sites on both DNA and histones lends plausibility to the likelihood that at least some of them may be influenced by alterations in metabolites or their by-products. Adding further support to this hypothesis, a number of direct links have been identified between the enzymes and processes responsible for alterations in chromatin structure, and metabolism. For example, both DNA methyl transferase enzymes (which actively methylate DNA) and histone methyl transferase enzymes require S-adenosyl methioinine (SAM), a product of 1-carbon (1C) metabolism, to carry out their functions. Flavin adenosine dinucleotide (FAD) and alpha-ketoglutarate (α-KG) are essential co-factors for the reverse reaction, removing the methyl groups (Iyer *et al*., 2009). FAD in its reduced state ($FADH_2$) carries energy; when oxidised, it is utilised in oxidation reactions within the tricarboxylic acid (TCA) cycle, also known as the Krebs or citric acid cycle, which forms a key part of aerobic respiration. Similarly, α-KG is an intermediate in the TCA cycle, and is derived from glutamine. Along with Fe^{2+}, it acts as a necessary co-factor for the TET family of enzymes, which actively remove methyl groups from DNA (Wossidlo *et al*., 2011). It is well documented that certain stressors, such as *in vitro* culture, during early development alter the metabolism of the embryo. Given the evidence discussed above, that both the maternal and paternal genomes are targets for active DNA demethylation by the TET family of enzymes in early development, it is perhaps no surprise that there are changes in DNA methylation status following stressors known to change metabolism (eg. *in vitro* culture (Stojanov and O'Neill, 2001; Morgan *et al*., 2008).

This interaction between metabolism and epigenetics is not unique to the methylation modification. Acetylation, recently shown to be as abundant as phosphorylation on the proteome, results from the covalent addition of an acetylCoA molecule to a lysine residue. When this modification occurs on histone tails, acetylation alters higher-order chromatin structure, whilst also serving as a docking feature for histone code readers (Choudhary *et al*., 2009). In mitochondria, acetylCoA is derived from citrate, which is in turn synthesised from glucose in the TCA. AcetylCoA, however, cannot cross the mitochondrial membrane, so for nuclear acetylCoA to be derived, citrate diffuses across the mitochondrial membrane, enters the nucleus via nuclear pores, and can then be converted to acetylCoA via adenosine triphosphate (ATP)-citrate lyase (ACL), promoting histone acetylation through increased substrate availability (Reytor *et al*., 2009; Wellen *et al*., 2009). Interestingly, there is a reduction of acetylation around genes critical for regulating glycolysis, resulting in a down-regulation of their transcription, and an inhibition of glycolysis, supporting intrinsic metaboloepigenetic interactions (Wellen *et al*., 2009; Martinez-Pastor *et al*., 2013). This provides an example of how "fuel-sensing" pathways involved in glucose metabolism can regulate chromatin, and in turn, transcription.

There is also a well-described influence of metabolism on the removal of acetyl groups by histone deacetylases (HDACs), which include a family/class of NAD^+-dependent enzymes, the Sirtuins (North and Verdin, 2004). NAD^+ accepts electrons from other molecules as it is reduced to NADH. This change in redox state is important for mitochondrial function, where it links the TCA cycle to the electron transport chain, with NADH donating the first electron for ATP production. The Sirtuin family of enzymes (HDAC Class III) are regulated by NAD+/NADH and tightly regulate glucose metabolism, such that *Sirt6* null mice have highly upregulated glycolysis, which triggers fatal hypoglycaemia at a young age (Zhong *et al*., 2010).

Interestingly, lactate, a product of metabolism in the post-compaction embryo, is also known to inhibit HDACs (Latham *et al*., 2012). With the tight dependence on availability of both acetylCoA and NAD^+ the presumption is that small changes in mitochondrial function, and/or alterations in redox status could dramatically alter the acetylation status of the histone landscape in the early embryo. Acetylation removes the positive charge on the histone, altering the interaction of the tails with the negatively charged DNA and relaxing the chromatin structure, promoting the transition from heterochromatin to euchromatin, and subsequently, transcription (Verdone *et al*., 2005). Given the importance of activation of the embryonic genome, and embryonic transcription during pre-implantation embryo development, it is conceivable that small perturbations to metabolism, like the ones seen during assisted reproductive technologies such as *in vitro* culture, and hormonal hyperstimulation, may alter the timing of development, as well as the activation of the embryonic genome.

Perhaps the most recently described and most poorly characterised epigenetic modification is that of O-linked glycosylation. O-linked glycosylation is a nutrient sensitive post-translational modification that involves the enzymatic addition of O-N-acetylglucosamine (O-GlcNAc) to serines and threonines in a manner akin to phosphorylation, on histone tails as well as a number of other chromatin-modifying proteins (Zhang *et al*., 2011). O-linked glycosylation has been demonstrated to target histone tails using the TET family of DNA-modifying enzymes, which are also metabolically linked (described above; Chen *et al*., 2013). While the function of glycosylation of histone tails is not yet well characterised, this O-linked glycosylation is also known to affect other key regulators of transcription including RNA polymerase II, where it competes with phosphorylation to modify the C-terminal domain (CTD), as well as targeting histonemethyl transferase and histone deacetylase complexes, and the repressive Polycomb complex (reviewed in Hanover *et al*., 2012). The addition of this molecule via the O-linked N-acetylglucosamine transferase (OGT) enzyme is regulated by flux of activity through the hexosamine biosynthesis pathway, which converts glucose to hexoses, and which is nutrient, or glucose sensing (Obici *et al*., 2002). This type of modification provides a direct mechanism by which availability of nutrients and metabolites can directly alter higher-order chromatin structure and organisation. In this way, alterations in nutrient availability, and modest changes in metabolism in the early embryo may dramatically alter the epigenetic landscape, and as such the trajectory of the embryo.

In addition to the covalent modifications that occur on the chromatin, non-covalent alterations, regulated by other chromatin remodelling complexes, are able to move, eject, or restructure nucleosomes. These use DNA-dependent ATPases, therefore requiring ATP as a co-factor for regulating transcription (Varga-Weisz, 2001). With sources of ATP switching from oxidative phosphorylation in early embryo development, to glycolysis later in development, it is not yet clear how these processes are regulated in the early embryo, but will no doubt be the focus of future research.

The interactome: is metabolism altering RNA storage and stability in the pre-implantation embryo?

Early embryonic stress can result in a number of poor outcomes, including but not limited to embryonic loss, deformity and defects and a range of adult onset disorders. However, the mechanisms by which stress negatively impacts the embryo long-term remain largely elusive. De novo transcription does not occur during oocyte maturation or the first cell cycle of the embryo in mouse. It is likely that the proteins required for the general reprogramming of the early embryo are translated from pre-existing mRNAs produced and stored during oogenesis. In particular, the zygote needs to switch from meiotic to mitotic divisions and to reprogram the haploid, specialised genome of the gametes into a totipotent diploid genome (Messerschmidt *et al*., 2014). Additionally, the zygote must resume the mitotic cell cycle, remodel the maternal and paternal chromatin (including the protamine-histone exchange in the paternal genome), activate transcription, and initiate the embryonic developmental program. Evidence suggests that maternal mRNAs are stored during oogenesis in an inactive state until they are recruited for translation (Oh *et al*., 2000). One mechanism by which this is proposed to occur is by elongation of the poly(A) tail (Piko and Clegg, 1982; Latham *et al*., 1991). It is very likely that other mechanisms exist, which may include unique RNA binding proteins, RNA modification and other complex nucleic acid interactions (ie. lncRNAs).

In spite of the importance of these early events, there is very little information detailing them in the early embryo, a likely consequence of the difficulties associated with working on a single, transcriptionally inactive cell. It is clear that a fine regulatory network controlling the spatial and temporal abundance of RNAs, as well as preventing RNA loss or premature translation must be present in the early embryo, to ensure reproductive success. In addition to the vulnerability of transcriptional inactivity, the pre-implantation embryo is in a window of epigenetic naivety, with the active and passive removal of the specialised germ-cell program in order to re-establish potency (Smith *et al*., 2014). Unable to respond by transcribing new pathways during “stressful” situations, the embryo is likely left to make the most of its active metabolic state, and mRNA reservoir, to adjust to

hostile situations (e.g. infection, hyperglycaemic stress, oxidative stress). This idea is supported by the "quiet embryo hypothesis", which proposes that the most developmentally competent embryo is that which has an efficient, but not high, metabolic activity (Leese, 2002). An example of this is *in vitro* culture; embryos derived *in vitro* are more metabolically active, and as such, more stressed than their *in vivo* counterparts.

Several enzymes that were once characterised only as metabolic enzymes have gone on to be described as RNA binding molecules. Enzymes involved in glycolysis and the pentose phosphate pathway including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aldolase, lactate dehydrogenase (LDH), phosphoglycerate kinase (PGK) and glucose-6-phosphate dehydrogenase (G6PDH) are now known to bind transcripts including but not limited to those of glucose transporters (McGowan and Pekala, 1996), immune regulators (GMCSF; Pioli *et al*., 2002), IFNα and IL2 (Nagy and Rigby, 1995) and tRNA and rRNA (Ryazanov, 1985; Singh and Green, 1993; reviewed in Ciesla, 2006). A landmark paper in the RNA field developed and utilised "interactome capture", a process of cross-linking all the RNAs within a cell at a given time to their protein partners. Using this technology, they categorised families of RNA binding proteins which had until recently been characterised only as functional metabolic enzymes (Castello *et al*., 2012). In addition to enzymes involved in carbohydrate, amino acid, lipid and nucleotide metabolism, eighteen of the RNA-binding enzymes used NAD^+, $NADP^+$, NADH, NADPH, FAD or $FADH_2$ as co-factors, and ten of these shared the ability to bind ATP and an anionic substrate such as succinate, L-aspartate or pyruvate (Castello *et al*., 2012). A RNA, enzyme and metabolite network hypothesis proposed that if these interactions were functionally relevant, then these proteins could broadly connect metabolism, RNA biology and post-transcriptional gene regulation.

This raises the possibility that under an altered metabolic state, either by pharmaceutical manipulation or in response to stressors, the embryo may employ the metabolic enzymes in their roles as RNA-binding proteins, to respond metabolically to the stress. This may result in the loss of stored RNA, or immature translation. This novel concept provides a mechanism by which an acute metabolic insult could negatively influence the trajectory of embryo development both in the short and longterm, by altering the control of RNA stability and translation.

Conclusion

It is a fascinating time for pre-implantation embryo research, with evidence from many fields of biology supporting important regulatory cross-talk between metabolism and epigenetics. With technological advances allowing single cell epigenome and metabolome analysis, the field of embryo metabologenomics is likely to flourish in the coming years. Understanding the mechanisms by which these two systems intertwine is likely to reveal the means by which gene-environment interactions regulate the transgenerational inheritance of health and disease, established during the first few days of life.

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Research challenges involving embryo pathogen interactions

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Abstract

In the late 1970s, embryo pathogen research was spawned. Initially, a great deal of funding was available to groups involved in embryo pathogen research. However, following years of research, and development of competent embryo processing procedures endorsed by the International Embryo Transfer Society (IETS), a growing belief developed that embryo transfer (ET) is innately safe and so funding for embryo pathogen research has since dwindled portentously. Even with continued growth of the ET industry and specifically with tremendous changes involving *in vitro* technologies, and recent outbreaks involving pathogens of concern, funding of ET research has not been a priority and/or a focus area for funding agencies for the last number of years. Funding issues are not the only challenge in embryo pathogen research but it is the primary challenge since no amount of research can be pursed without funding. Some of these additional challenges include; a large number and variety of pathogens which need to be systematically investigated including new and re-emerging pathogens, utilization of animal origin products which have the potential to harbor and transmit pathogens, the ability of pathogens to adapt and change to their hosts and environment resulting in variation of affinity and virulence, reliable testing of these pathogens and trained personnel to perform studies, collect and interpret data and to knowledgeably handle pathogens that have zoonotic pathogens. This paper reviews these challenges facing embryo pathogen research today.

Keywords: embryo pathogens, *in vitro* produced embryos, *in vivo* derived embryos.

Introduction

The techniques used for nonsurgical embryo recovery; including collection, cryopreservation, and nonsurgical transfer of *in vivo* derived (IVD) bovine embryos, rapidly evolved into a frequently utilized commercial procedure. Embryos then became available for international commerce. This created concerns of diseases, which might be transmitted inadvertently through embryo transfer (ET). It stimulated establishment of protocols to ensure removal and hence, prevention of pathogen adherence to embryos. Also, standardized regulations involving embryo movement were also constructed to further minimize any potential pathogen transmission. A tremendous amount of research has been completed to test these protocols and procedures. There is a great deal of confidence in the standard processing procedures which has in some cases has resulted in complacency in embryo pathogen research. However, more research is essential to ensure the safety of transfer of IVD embryos, *in vitro* produced (IVP) and cloned embryos as well. The objective of this paper is to discuss the current challenges involving embryo pathogen research.

Research development, regulations and funding

Since the late 1970s, the concern of inadvertent disease transmission via embryos and resultant infectious disease outbreaks following in suite spawned the need for research involving embryo pathogen interactions. Specifically, it was deemed that international movement of embryos needed to be regulated to prevent unwanted and foreign animal diseases from gaining entrance to countries currently listed as free from these regulatory diseases. In the 1970's through to the 1990's there was a significant amount of funding available for embryo pathogen research provided by government entities such as the United States Department of Agriculture and other regulatory bodies in other countries.

Initially there was an absence of uniform import requirements, which lead to importing countries developing expensive, often times cumbersome and sometimes arbitrary embryo-health certifying procedures. International regulations required herds and/or nations in which donors resided to be free of all diseases of potential concern to importing countries (Waters, 1981). Other regulations consisted of embryo donors being housed in isolation facilities for an acceptable period then to be tested and certified to be free from an assortment of infectious diseases. Additionally, the embryo recipient herds were required to be isolated throughout pregnancy in the country of destination and following birth they and their offspring where similarly tested to confirm absence of a variety of diseases. Hence, there was a tremendous need to develop sanitary collection and processing procedures, which could function as "broad spectrum" health certifying procedures. However, little research and data was available to determine what was necessary and little

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experimental evidence to confirm their beliefs (Atwell, 1987). This situation was producing an environment that would seriously undermine international movement of embryos. This state provided the stimulus necessary for gaining the funding to conduct essential research. Specifically, the research was needed to test the belief that zona pellucida-intact (ZP-I), IVD, bovine embryos were not likely to transmit infectious diseases if properly handled and processed even if some donors resided in "infected" countries.

Experimental approaches

The primary experimental hypotheses was that if the embryo had an intact zona pellucida and the embryos where properly handled it would protect the early conceptus from infectious agents and transmission of embryos would be minimal (Stringfellow *et al*., 2004; Givens *et al*., 2007). The null hypothesis was that diseases might be transmitted by transfer of embryos. It was determined in order to provide consistency in research so that data might be comparable, four main approaches where outlined and utilized (Bowen *et al*., 1978; Archbald *et al*., 1979; Hare, 1990). In the first approach, recovered ZP-I embryos from pathogen free donors were artificially exposed *in vitro* to a variety of pathogens that were of domestic or international concern. The embryos were subjected to cleaning protocols and then subsequently tested *in vitro* for the pathogen in question. The second approach was very similar with the exception that following experimentally exposure of the embryo to the pathogen or pathogens, the embryos were transferred to disease-free (sentinel) recipients. The recipients were subsequently observed and tested along with their offspring for presence of the agent. The third approach was to collect ZP-I embryos from infected donors, clean them, and subsequently, assay the embryos for pathogens. The fourth approach was to again collect the ZP-I embryos from infected donors and then transfer the cleansed embryos to disease-free (sentinel) recipients which along with their offspring were then observed and tested for the disease agents. The goal was that each pathogen of concern would undergo this comprehensive series of experiments using each of the four approaches. Hence, the data gathered from these studies involving the specific pathogens analyzed would be essentially conclusive and the likelihood of transmission of the pathogen via ET would be known.

Although, this plan seemed to be comprehensive there are shortcomings revolving around the reliability of the testing available. Also, the correct controlling of variables and the interpretation of the evidence presented within the population tested are always of concern. Although, there has been a decline in the number of new research studies involving embryos over the past decade, there has been a tremendous increase in number and type of technologies available for determining the presence of a pathogen or pathogens in culture systems. Additionally, the sensitivity of the available assays has also increased dramatically. The RT-q-PCR utilized in the above-mentioned studies is representative of the ability of current assays to determine the presence of specific disease agents to the point that the levels detected may be below what would actually constitute an infective dose. The development of numerous assays used for screening, detection, and quantification of specific pathogens has been prolific. Assays have been developed for a number of reasons including to be utilized in eradication programs and/or to be utilized as a research tool for those pathogens of greatest concern such as BVDV and BHV-19 (Marley *et al*., 2008; Gard *et al*., 2009; Gregg *et al*., 2009). However, these techniques require appropriate trained individuals with careful attention to detail in order to prevent false negative or false positive results leading to false conclusions.

Pathogens evaluated

A variety of pathogens has been studied, but there is a multitude which have not been, and more information is needed. A greater emphasis for study was placed on those of regulatory concern in either domestic or international commerce, including bluetongue virus, bovine spongiform encephalopathy, *Brucella abortus*, enzootic bovine leukosis virus, foot and mouth disease virus, and infectious bovine rhinotracheitis virus (bovine herpesvirus-1). Aujeszky's Disease in swine and the scrapie agent in sheep were also the objects of thorough study. The embryo washing procedures originally developed and validated in numerous studies provided the basis for the commonly accepted embryo processing procedures (a.k.a. embryo washing and trypsin treatment), which are recommended today for health certification of IVD embryos (Bowen *et al.*, 1979; Singh *et al.,* 1987; Thibier and Nibart, 1987; Stringfellow and Wright, 1989; Wrathall, *et al.*, 1995, Stringfellow, 2010).

Additionally, many of the pathogens which were evaluated early on were laboratory strains, which can be very different from wild strains. Certain pathogens mutate during replication on a regular basis, such as bovine viral diarrhea virus (BVDV), which mutates every time it replicates. Hence, the early experiments utilizing the laboratory strains did not evaluate a good spectrum of BVDV strains. Reports by Lindberg and Drew (Lindberg *et al*., 2000; Drew *et al*., 2002) stimulated further investigations to the possibility of transmission of BVDV via ET due to sero-conversion of heifers after embryo transfer and birth of a PI calf following ET, respectively. Contaminated Fetal Bovine Serum (FBS) was thought to be the inciting cause of these infections. Hence, additional studies were necessary to determine if different strains of BVDV would remain associated with BVDV following

standard IETS processing procedures for IVD embryos and whether this associated virus could be transmitted via ET. Studies were performed which highlighted the variation in affinity between different strains of BVDV and the affinity of these strains for embryos (Waldrop *et al*., 2004a, b). Some strains were shown to maintain association with embryos following IETS processing procedures including when trypsin treatment was added to the processing procedures (Waldrop *et al*., 2004a, b). These high affinity strains became more of a concern when it was determined that the embryo-associated virus was indeed infective in both *in vitro* and *in vivo* studies (Waldrop *et al*., 2004b, 2005). Current research by Gard *et al*. (2009) reported that 27% of IVD and 42% of IVP embryos had embryo-associated virus (EAV) following artificial exposure to a high affinity strain of BVDV (SD-1, type 1a) following washing procedures in accordance with IETS (without trypsin). It was also found that the range of the amount of EAV was 100 to 450 cell culture infective doses to the 50% endpoint ($CCID_{50}$)/embryo (Gard *et al*., 2009). In previous studies, EAV was also determined to be infectious in an *in vitro* culture system and in an *in vivo* model and through intravenous inoculation of embryos and then in an intrauterine inoculation of embryos and 898 ($CCID_{50}$) of BVDV (SD-1, type 1a) into virus negative and seronegative recipients (Waldrop *et al*., 2004b, 2005; Gard *et al*., 2009, 2010). It was found that all recipients of embryos and virus became viremic, and then seroconverted (Waldrop *et al*., 2005; Gard *et al*., 2009, 2010).

The finding that no BVDV positive offspring were produced in these studies is similar to the results found Bielanski *et al*. (1998). In this study *in vitro*-produced embryos were exposed to noncytopathic biotypes of BVDV for 1 h, type 2 strain (P-131) or a type 1 strain (NY-1), and then washed in accordance with IETS guidelines (no trypsin treatment) and then transferred to seronegative BVDV negative recipients. However, none of the recipients following intrauterine transfer of embryos exposed to the type 1 strain seroconverted but of the 35 recipients receiving embryos exposed to the type 2 strain 18 seroconverted (51%), and there were 11 pregnancies at 30 days post transfer but of these only two resulted in live offspring. These two offspring were determined to be BVDV negative and seronegative. Hence, the infection seemed not to be recognized by the fetus and/or the virus was destroyed prior to development of immune competence so in fact no antibodies were formed by the fetus. The results of no detectable seroconversion from the type 1 strain and abortion due to the type 2 BVDV may be the results of mutations within the virus, or the test applied might have had produced false negatives. In a study by Meyers *et al*. (2007), decreases in interferon production, abortion and presence of virus in fetal tissues did not result when pregnant cattle were injected with two different mutant strains of virus. Each of these mutant strains had mutations specifically affecting both the N-terminal protease (N(pro)) and the deletion of codon 349, which abrogates the RNAse activity of the structural glycoprotein E(rns; Meyers *et al*., 2007). However, decreases in interferon production, abortion and presence of virus in fetal tissues did occur with wildtype viruses and in viruses in which only one mutation of either N(pro) or E(rns) occurred (Meyers *et al*., 2007). Therefore, the establishment of persistent infections requires both N(pro) and E(rns). It is logical to ascertain that the type 1 strain utilized in Bielanski's study might have had mutations within these areas, resulting in adequate interferon production and no fetal infection. Each specific strain may affect interferon production differently and therefore may or may not result in fetal infection, and that fetal infection may or may not be fatal. A thorough evaluation is important as the ability to transmit BVDV via an embryo would necessitate re-evaluation of embryo health certification procedures along with implementation of additional regulations on embryos exported from BVDV-positive countries to those countries where BVDV has been eradicated. So, BVDV is a good example of challenges facing embryo pathogen research namely intra-pathogen mutations and variation.

Classical Scrapie and Atypical Scrapie pose another example of intra-pathogens variations, which may confound research findings when they are treated as the same. Studies have established that Classical Scrapie may be transmitted within and between flocks by various routes and that transmission when utilizing ET may occur (Detwiler and Baylis , 2003 Andréoletti *et al*., 2011). However, secondary cases involving Atypical Scrapie are rare (Fediaevsky *et al*., 2009, 2010; Garza *et al*., 2011) and a low transmissibility has been seen between sheep in case controlled studies (Fediaevsky *et al*., 2009, 2010; Garza *et al*., 2011) but further assessment are being performed (Hopp *et al*., 2006). Additionally, Atypical Scrapie is only detectable in brain tissue and not in lymphoreticular system so it is assumed to have a decreased risk of transmission via ET when compared to that of Classical scrapie (Wrathall *et al*., 2008; Garza *et al*., 2011; Ligios *et al*., 2011). Classical Scrapie has been detected in fetuses of scrapie-affected ewes (Nicholson *et al*., 2008; Garza *et al*., 2011) and semen from Scrapie-infected rams (Rubenstein *et al*., 2012) making the infection of embryos a significant possibility if these embryos are not processed in accordance with the IETS guidelines for IVD embryos (Stringfellow *et al*., 2010). However, if embryos are processed in accordance with IETS standards for IVD embryos than there would be a negligible chance for transmission involving Classical Scrapie in sheep, as it is a category 1 disease according to the OIE (Stringfellow *et al*., 2010). The OIE code chapter 4.7.14 (World Organisation for Animal Health - OIE *et al*., 2014a, b) does not includes Atypical Scrapie because it is known to be different than Classical

Scrapie. Hence, it has been suggested to the OIE by the HASAC subcommittee, that Atypical Scrapie should be described as a category 3 disease since more research is necessary to completely determine the risk of transmission even though it appears on evaluation to have less potential for transmission via embryos.

In vitro produced embryos

Furthermore, when evaluating *in vitro* produced (IVP) embryos verses IVD embryos there are clear differences such as differences in the zona pellucida, the potentially-contaminated materials of abattoir-origin that have been regularly utilized in IVF embryo production, and the multiple steps that might result in in advertent contamination (Stringfellow and Wrathall, 1995). Therefore, the potential to introduce bacterial and viral contaminants during the process of producing IVP embryos has been emphasized in many studies (Stringfellow Wrathall, 1995; Bielanski and Jordan**,** 1996; Givens *et al*., 1999, 2001, 2002; Gard *et al*., 2009). The more porous nature of the zona pellucida of IVP bovine embryos was evident by the results of those studying a number of viruses such as: bluetongue virus, bovine viral diarrhea virus, and foot and mouth disease. It was found that virus adhered to the ZP of the IVP embryos while they had not adhered to the ZP of the of the bovine IVD embryos (Marquant-Le Guienne *et al*., 1998; Stringfellow *et al*., 2004). It continues to be clear that embryo washing, while beneficial in reducing environmental pathogen load, would not be as universally reliable for certifying the health of IVP embryos and can result in confounding results in studies due to contamination. So, it is necessary that additional steps are taken when analyzing pathogens of IVP embryos such as: (1) establishing minimum standards for sanitation in the laboratory and handling of oocytes; (2) pre- testing of animals utilized for OPU and materials of animal origin for specific pathogens and contaminants; (3) the judicious use of antimicrobials; (4) continuous, follow-up testing for contaminants of samples from IVM, IVF and IVC cultures; (5) washing of oocytes and developed embryos using the techniques applied to IVD embryos as a complementary control measure to reduce environmental pathogen load; and (6) when possible utilize synthetic oviductal media to minimize contaminants in the system. Details of processing IVP and micromanipulated embryos to remove and prevent contamination are outlined by the OIE chapter 4.8, and 4.9. (OIE, 2014c, d).

Somatic cell nuclear transfer

The most recent embryo method for *in vitro* production of bovine embryos, identified as somatic cell nuclear transfer (SCNT) shares the same concerns that IVP embryos (Stringfellow *et al*., 2004), do except some added caveats exist. The first major concern would be the removal or at least fracture of the zona pellucida. Additional, avenues for contamination exist such as the long duration of cell culture lasting weeks to months allows for exponential chances of extraneous pathogens interfering within the system. Hence, strategies for health certification of resulting embryos have not focused on any specific methods for washing the embryos, but rather, they have focused on risks for introduction of infectious agents and testing protocols to certify that the embryos or materials of animal origin used in their production are specific-pathogen-free prior to transfer of the embryos or their movement in commerce (Stringfellow *et al*., 2004). This is imperative when utilizing SCNT embryos in studies so that variables can be managed within the study.

However, there is a continued false sense of security with IVD embryos, IVP embryos, as well as cloned embryos. The argument has been that since no major outbreaks have been traced back to ET, so, it must not be a viable problem. Hence, this security has resulted in complacency of funding agencies and embryo pathogen research is no longer listed as a focus area even in the face of recent outbreaks and studies which highlight a need for additional embryo pathogen research. Some believe that sufficient research has been completed and that even with increased international embryo movement, especially with IVP embryos, no steps should be taken to stimulate revenue for additional studies. This seems to be a short-sighted approach since IVP embryos are of particular concern because the standard processing procedures are not as affective at removal of pathogens. Also, evolution of pathogens is a constant process resulting in re-emerging pathogens and formation of new pathogens that have not been fully tested. In order for the ET industry to continue to have continued growth, additional research is necessary. There is a desperate need to increase research in embryo pathogen interactions to provide up to date information on the diseases of concern and to elevate commerce and the efficiency of the embryo production systems.

Zoonotic pathogens

Additionally, public health concerns can be raised with some pathogens, especially those that are zoonotic. A good example of this is seen with *Coxiella burnetti*, which has been detected in media from embryo collections and uterine tissue samples from goats (Alsaleha *et al*., 2013). In recent studies by Alsaleh *et al.* (2013, 2014*)*, they reported that *C. burnetti* was not removed when IVD caprine embryos and/or IVP bovine embryos were exposed *in vitro* and subsequently underwent standard washing procedures. The pathogen, *C. burnetti,* is zoonotic. Hence, handling of the donor and potential recipient contamination might result in human infection. Thus, appropriate handling of the donor, recipient and the embryos is necessary so as not

to institute disease in the researchers themselves.

Summary

There are many areas that are challenging to embryo pathogen research. The most primary is lack of funding. Since research requires funding, it is a guarantee that studies cannot be performed without it. Today, funding agencies do not list embryo pathogen research as a focus area even in the face of recent outbreaks and studies which highlight a need for additional embryo pathogen research. Embryo movement in commerce will be hampered if further research is not performed. Research is the key to increase the safety and efficacy of the embryo transfer industry. Additional challenges include; the large number and variety of pathogens, new and re-emerging pathogens, utilization of animal origin products, which often harbor and transmit pathogens, adaption of pathogen to their hosts and environment resulting in variation of affinity and virulence, reliable testing of these pathogens and trained personnel to perform studies, collect and interpret data, international regulations and the zoonotic potential of some pathogens.

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The infectious disease epidemiologic triangle of bovine uterine diseases

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Abstract

Postpartum uterine diseases are important for animal welfare and economic reasons, causing cow discomfort, elimination from the herd and impaired reproductive performance. Metritis is characterized as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge within 21 days after parturition. Endometritis is defined as inflammation of the endometrium after 21 days postpartum without systemic signs of illness, and can be considered the chronic stage of uterine inflammation. It has been reported that the metritis affects 10 to 20% of cows, and endometritis affects 5.3 to 52.6% of cows. Metritis affects the cow systemically, and has a negative impact on milk production and reproductive performance. Cows affected with endometritis are not systemically ill, and do not have their milk production altered; however, they have impaired reproductive performance. Metritis and endometritis are complex multifactorial diseases, and a wide range of factors contributes to their occurrence. They are often associated with mixed bacterial infection of the uterus, and the major pathogens associated with uterine diseases are *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum.* Events during the transition period related to negative energy balance and metabolic imbalance, mineral deficiencies, leading to immunosuppression are of great important during establishment of intrauterine bacterial infections. This, combined with endometrium trauma events during parturition (such as calving related problems), and environmental factors (poor hygiene at calving, housing type and calving season), increases the risk of metritis and endometritis.

Keywords: dairy cows, endometritis, metritis, reproduction, uterine diseases.

Introduction

The infectious disease epidemiologic triangle illustrates the interaction of epidemiologic factors that contribute to the outbreak of an infectious disease: the host, the pathogen or disease-causing organism, and the environment (Merrill, 2013). Metritis and endometritis are complex multifactorial diseases caused by mixed bacterial infection. During the past decades, several studies contributed to better understanding of the factors associated with the host, the pathogens, and the environment on how these factors influence the risk of uterine diseases. The objective of this review is to enumerate and discuss the published data on many factors that predispose to the development of uterine diseases in dairy cows.

Introduction to uterine diseases of dairy cows

Reproductive efficiency is a trait of great importance for the modern dairy industry, affecting the overall economic outcome of dairy enterprise. A healthy reproductive tract after parturition is essential for a satisfactory reproductive performance. Postpartum uterine diseases are important for animal welfare and economic reasons, causing cow discomfort, elimination from the herd and impaired reproductive performance. In North America, puerperal metritis affects 10 to 20% of cows (LeBlanc *et al.*, 2011), whereas the incidence of endometritis is approximately 28%, ranging from 5.3 to 52.6% (Dubuc *et al.*, 2010a; Cheong *et al.*, 2012).

Metritis is characterized as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge within 21 days after parturition; however, the metritis incidence peaks within the first week post-partum. When metritis is associated with signs of systemic illness (decreased milk yield, dullness, or other signs of toxemia) and temperature >39.5°C, the appropriate term is puerperal metritis. Approximately half of the metritic cows are not diagnosed with fever (Benzaquen *et al.*, 2007; Martinez *et al.*, 2012; Lima *et al.*, 2014).

The effects of metritis on productivity are striking. Metritis has a detrimental effect on milk production during early lactation (Rajala and Grohn, 1998; Huzzey *et al.*, 2007; Giuliodori *et al.*, 2013), especially for multiparous cows (Dubuc *et al.*, 2011; Wittrock *et al.*, 2011). Metritis also contributes to reproductive failure, as cows diagnosed with metritis have decreased conception rate (Overton and Fetrow, 2008; Giuliodori *et al.*, 2013). Data regarding the effect of metritis on survivability are inconsistent; studies have reported no effect of metritis on culling rate (Rajala and Grohn, 1998; Dubuc *et al.*, 2011), whereas others observed that cows diagnosed with metritis are more likely to leave the herd than healthy cows (Linden *et al.*, 2009; Wittrock *et al.*, 2011). Wittrock *et al.* (2011) suggested that multiparous cows affected by metritis

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were at increased risk of being culled, primarily because of the detrimental effect of disease on milk production, rather than reproductive failure. Metritis is frequently treated with systemic antibiotic therapy. The antibiotics of choice to treat metritis are ceftiofur or penicillin (Smith *et al.*, 1998; Drillich *et al.*, 2001); however, alternative treatment with ampicillin had similar efficacy to ceftiofur (Drillich *et al.*, 2003; Lima *et al.*, 2014). The economic losses caused by each metritis case have been calculated at approximately US$329-386 due to antibiotic treatment and the detrimental effects of metritis on reproductive performance, milk production, and survivability (Drillich *et al.*, 2001; Overton and Fetrow, 2008).

Endometritis is defined as inflammation of the endometrium after 21 days postpartum without systemic signs of illness, and can be considered the chronic stage of uterine inflammation. Endometritis has been classified as clinical or subclinical. Clinical endometritis is characterized by the presence of purulent or mucopurulent uterine exudates detectable in the vagina after 21 days postpartum (Sheldon *et al.*, 2006). Subclinical endometritis is defined as the inflammation of the endometrium determined by cytology of samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of purulent discharge in the vagina (Gilbert *et al.*, 2005). Although the definition of clinical endometritis is largely accepted and used by clinicians and researchers, a recent study challenged assumptions of this method of diagnosis, showing that cows with purulent vaginal discharge (PVD) did not always present endometrial inflammation; the nomenclature PVD has been proposed to properly represent what have been diagnosed in cases of clinical endometritis (Dubuc *et al.*, 2010a). However, in this literature review, we will use the terminology clinical endometritis. To define subclinical endometritis, various cutoff points of neutrophils in uterine cytology have been used, depending on stage of lactation that samples were collected. Increased cutoff points were used to define uterine inflammation in earlier stages of lactation. For instance, subclinical endometritis was defined as the presence of neutrophils in uterine cytology exceeding 18 and 10% relative to total cell count, for samples collected at 20 - 33 days and 34 - 47 days postpartum, respectively (Kasimanickam *et al.*, 2004). Others have used 5% of neutrophils in uterine cytology as the cutoff point used to define subclinical endometritis (Gilbert *et al.*, 2005; Lima *et al.*, 2013). Recent studies have been using the terminology cytological endometritis instead of subclinical endometritis; cytological endometritis is defined as the inflammation of the endometrium determined by cytology, regardless of the presence of clinical endometritis (Dubuc *et al.*, 2010a; Cheong *et al.*, 2012; Yasui *et al.*, 2014). Several diagnostic methods have been used to evaluate the reproductive tract infection and inflammation in dairy cows, such as vaginoscopy (Studer and Morrow, 1978; Barlund *et al.*, 2008; Westermann *et al*., 2010), the metrickeck device (McDougall *et al.*, 2007; Brick *et al.*, 2012; Machado *et al.*, 2015), ultrasonography of uterus and cervix (Senosy *et al.*, 2009; Brick *et al.*, 2012; Polat *et al.*, 2015), intrauterine bacterial culture (Studer and Morrow, 1978; Westermann *et al.*, 2010), uterine biopsy (Bonnett *et al.*, 1991; Meira Jr. *et al.*, 2012), reagent strips used to measure leukocyte esterase, protein and pH of uterine lavage samples (Cheong *et al.*, 2012), uterine lavage samples optical density (Machado *et al.*, 2012b), and cytology (Gilbert *et al.*, 2005; Dubuc *et al.*, 2010a; Lima *et al.*, 2013). To assess and evaluate the validation of each diagnostic method is beyond the objectives of this review, and has been intensively reviewed (de Boer *et al.*, 2014).

Differently from metritis, endometritis is not accompanied by systemic symptoms, being a disease contained within the uterus. Although it has been reported that endometritis does not directly impact milk production (Erb *et al.*, 1985; Dubuc *et al.*, 2011), others have shown that primiparous cows that produced more milk, and multimaprous cows that produces less milkin the first month of lactation were more likely to develop subclinical endometritis (Cheong *et al.*, 2011; Galvão *et al.*, 2010). However, endometritis impairs reproduction (Gilbert *et al.*, 2005; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), and as a consequence has a negative economic impact on the modern dairy industry (Lee and Kim, 2007). It has been reported that clinical and subclinical endometritis reduce conception rate (Galvão *et al.*, 2009; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), increase the calving-to-conception interval (Barlund *et al.*, 2008; Dubuc *et al.*, 2010a; Machado *et al.*, 2015), and increase embryonic mortality (Lima *et al.*, 2013; Machado *et al.*, 2015). To date, many endometritis therapy strategies have been evaluated with controversial efficacy, such as intrauterine administered chlorhexidine (Gilbert and Schwark, 1992), enzymes (Drillich *et al.*, 2005), hypertonic dextrose (Brick *et al.*, 2012; Machado *et al.*, 2015), and the systemic administration of PGF2α (LeBlanc *et al.*, 2002; Kasimanickam *et al.*, 2005; Lima *et al.*, 2013). Although the intrauterine infusion of cephalosporin has been reported to be efficacious to treat clinical endometritis (Runciman *et al.*, 2008; McDougall *et al.*, 2013), the use of intrauterine administered antibiotic is currently not approved in the

The host

Transition period, metabolic imbalance, mineral deficiency and immunosuppression

The transition period (defined as the period from 3 weeks before to 3 weeks after calving) is extremely challenging for the dairy cow (Drackley, 1999). As the time of calving approaches, the nutrient

requirements for fetal growth increase to maximum levels, whereas the dry matter intake (DMI) decreases approximately 20% (Bell, 1995). Around parturition, cows have to deal with nutritional changes, because their diet changes abruptly from being forage-based to concentrate-rich diets. They also dramatically alter their metabolism to supply the mammary gland with nutrients necessary for milk synthesis (Bell, 1995; Goff *et al.*, 2002). However, the nutrient requirements for milk synthesis during the first weeks of lactation exceeds nutrient intake. To support milk production, the cow has to mobilize her body reserves, leading to a condition of negative energy balance (NEB; Roche *et al.*, 2009). Dairy cows undergo a state of insulin resistance during early lactation, reducing glucose uptake by body tissues, helping to meet the nutrient demands for milk production during the first weeks of lactation (Bell, 1995). Combined with insulin resistance, a down-regulation in the liver growth hormone (GH) receptors have also been reported, which leads to a reduction in circulating insulin-like growth factor (IGF) and increased the circulating GH, resulting in increased lipolysis (Lucy *et al.*, 2001; Wathes *et al.*, 2009). At parturition, the blood progesterone level falls drastically, followed by a temporary increase of blood concentrations of estrogen and glucocorticoids, contributing to decreased DMI and mobilization of body fat reserves (Drackley *et al.*, 2005; Ingvartsen, 2006).

The complex changes during the transition period lead to a state of metabolic imbalance, which results in exacerbated fat mobilization and body condition score loss during early lactation (Roche *et al.*, 2007). This reflected in elevated circulating concentration of non-esterified fatty acids (NEFA; Kunz *et al.*, 1985; Busato *et al.*, 2002). NEFA is an excellent source of energy for many body tissues and is also used for milk fat synthesis. However, when the liver meets its ATP needs, the uptake of NEFA to complete β-oxidation is diverted to β-hydroxybutyrate (BHBA) and other ketone bodies (Drackley, 1999). Additionally, when in high concentration, NEFA can be resterified into triglycerides and accumulate in the liver causing a condition known as fatty liver (Strang *et al.*, 1998).

The mechanisms by which all these factors associated with NEB and metabolic imbalance will contribute to a state of immunosuppression during the periparturient period that not yet fully understood. During this period, impairment of polymorphonuclear neutrophils (PMN) function and decreased blood concentration of immunoglobulins are observed (Kehrli *et al.*, 1989; Hoeben *et al.*, 2000; Colitti and Stefanon, 2006; Sordillo *et al.*, 2007; van Knegsel *et al.*, 2007; Herr *et al.*, 2011). Reduced DMI and elevated concentration of NEFA and BHBA are associated with immunosuppression during the transition period (Rukkwamsuk *et al.*, 1999; Hammon *et al.*, 2006; Graugnard *et al.*, 2012). *In vitro* studies have shown that bovine PMNs incubated with elevated concentration of NEFA have impaired function and viability (Lacetera *et al.*, 2004; Scalia *et al.*, 2006). High concentration of BHBA reduced bovine PMN capacity for chemotaxis, oxidative burst, and phagocytosis (Klucinski *et al.*, 1988; Hoeben *et al.*, 1997; Suriyasathaporn *et al.*, 1999). Recently, it was demonstrated that an induced hyperketonemia in cows disturbed the mammary gland immune response to lipopolysaccharide (LPS) challenge (Zarrin *et al.*, 2014). Cows undergoing severe NEB have decreased circulating concentration of IGF-1 during the early postpartum period (Lucy *et al.*, 2001; Wathes *et al.*, 2009). The bovine endometrium expresses the IGF system genes (Llewellyn *et al.*, 2008), which play a role in tissue repair, promoting proliferation and healing during uterine involution (Wathes *et al.*, 2011), and alters endometrial gene expression related to immune responses (Wathes *et al.*, 2009). Additionally, natural antibodies (NAb), an important component of the humoral branch of the innate immune system (Avrameas, 1991), have been reported to have a negative association with elevated serum NEFA concentrations (van Knegsel *et al.*, 2007, 2012).

The relationship between NEB, followed by metabolic imbalance during the periparturient period, and uterine diseases is well established in the current literature. For instance, feeding behavior and DMI has been associated with metritis and endometritis. Compared to healthy animals, cows diagnosed with metritis or endometritis had less feeding time and decreased DMI during the transition period (Urton *et al.*, 2005; Hammon *et al.*, 2006; Huzzey *et al.*, 2007), and had increased BCS loss during the dry period (Markusfeld *et al.*, 1997; Kim and Suh, 2003). The incidence of endometritis was increased for cows with low BCS at calving (Hoedemaker *et al.*, 2009; Dubuc *et al.*, 2010b), whereas overconditioned cows are at increased risk of developing metritis (Kaneene and Miller, 1995).

Although there are some minor discrepancies in the literature, generally, cows that develop metritis or endometritis have elevated circulating concentration of NEFA in the week preceding parturition, and elevated NEFA and BHBA serum concentration in the first week of lactation (Hammon *et al.*, 2006; Dubuc *et al.*, 2010b; Galvão *et al.*, 2010; Ospina *et al.*, 2010). These parameters have been explored as diagnostic tools to identify cows at high risk of developing uterine diseases, with satisfactory accuracy (Dubuc *et al.*, 2010b; Ospina *et al.*, 2010; Giuliodori *et al.*, 2013). Recently, it was reported that high prepartum IGF-1 was associated with reduced risk of developing metritis or other postpartum diseases (Piechotta *et al.*, 2012; Giuliodori *et al.*, 2013).

This metabolic imbalance experienced by cows during the transition period is thought to increase the production of reactive oxygen species (ROS). Combined with reduced anti-oxidant capacity during the

periparturient period, cows experience a condition called oxidative stress (Castillo *et al.*, 2005; Sordillo, 2005; Abuelo *et al.*, 2013). Additionally, the blood concentrations of some minerals, such as Ca, P, Zn, and Cu are affected with the onset of lactation, as the blood minerals are utilized by the mammary gland for milk production (Goff and Stabel, 1990; Xin *et al.*, 1993; Meglia *et al.*, 2001; Goff *et al.*, 2002). The immune system is also suppressed by the transient minerals deficiency experienced by cows in the weeks around parturition, especially hypocalcemia (Ducusin *et al.*, 2003; Martinez *et al.*, 2012, 2014). Associations between low blood Ca concentration around parturition and compromised neutrophil phagocytosis and oxidative burst activities have been reported (Kimura *et al.*, 2006; Martinez *et al.*, 2012). It was proposed that the impairment of phagocytosis and oxidative burst activities in cows undergoing hypocalcemia could be explained by the fast decline of cytosolic iCa^{2+} (Martinez *et al.*, 2014). Furthermore, low blood Se concentration has been associated with impaired neutrophil adhesion, migration, and killing ability (Ndiweni and Finch, 1995; Cebra *et al.*, 2003). Deficiency of Cu and Zn is also linked to impaired immunity (Shankar and Prasad, 1998; Spears and Weiss, 2008).

Decreased postpartum concentration of blood minerals is associated with uterine diseases (Martinez *et al.*, 2012; Bicalho *et al.*, 2014a). Hypocalcemia after parturition was associated with increased incidences of metritis and clinical endometritis (Martinez *et al.*, 2012; Bicalho *et al.*, 2014a), and decreased postpartum serum concentrations of P, Zn, Cu, Mo and Se were reported to be linked with metritis and clinical endometritis (Bicalho *et al.*, 2014a). Injectable supplementation with a product containing Cu, Se, Zn, and Mn during the dry period decreased the incidence of clinical endometritis, and the presence of known intrauterine pathogens, suggesting that some of these trace minerals could be playing a protective role in the postpartum intrauterine environment (Machado *et al.*, 2012c, 2013).

During the pregnancy, the immune function of the uterus is suppressed to avoid maternal immune responses against the allogeneic conceptus. This uterine immunosuppression is partially regulated by elevated concentration of progesterone during the pregnancy (Padua *et al.*, 2005). Maternal tolerance to the fetus is also possible because of inhibition of inflammatory responses mediated by T regulatory cells (Lee *et al.*, 1992; Aluvihare *et al.*, 2004). This, combined with the systemic immunosuppression faced by dairy cows during the transition period discussed earlier, makes the uterus very susceptible to diseases in the early postpartum period. Associations between metritis and endometritis, and suppressed periparturient immune system have been reported in several studies. Although the data regarding the association between PMN phagocytic activity and uterine diseases is inconsistent (Mateus *et al.*, 2002; Kim *et al.*, 2005; Machado *et al.*, 2013), associations between the killing ability of neutrophils are more consistent. It was observed that cows that developed metritis have neutrophils that produced less superoxide activity before parturition (Cai *et al.*, 1994), and that decreased blood PMN oxidative burst activity is associated with increased risk of developing endometritis (Mateus *et al.*, 2002). The peripheral PMN killing ability determined by myeloperoxidase activity and cytochrome c reduction activity is reduced on the day of calving in cows that developed metritis and subclinical endometritis (Hammon *et al.*, 2006). Energy status of blood PMN measured by PMN glycogen concentration was also associated with uterine disease; cows that developed metritis or subclinical endometritis have lesser blood PMN glycogen than healthy cows (Galvão *et al.*, 2010). Recently, it was suggested that decreased circulating NAb concentration is another factor that may contribute to the impairment of the innate immune system around parturition, increasing the risk of uterine diseases (Machado *et al.*, 2014a).

Physical factors and genetic parameters

There are several risk factors that contribute to postpartum uterine contamination or physical damage of the uterine tissue, such as retained placenta (RP), calving abnormalities (dystocia, twins, and stillbirth), angle of the vulva, and parity. Many of these factors, combined with metabolic health parameters, were used to build a model aiming to predict postpartum diseases, including metritis (Vergara *et al.*, 2014).

Several studies have shown that RP is one of the most important risk factors for metritis and endometritis in dairy cows (Erb *et al.*, 1985; Kaneene and Miller, 1995; Bruun *et al.*, 2002; Machado *et al.*, 2012b). Retained placenta contributes to development of uterine diseases because cows that have their fetal membranes retained are immunosuppressed, have more uterine tissue damage (Paisley *et al.*, 1986), and are more likely to allow bacterial growth in the uterine lumen (Paisley *et al.*, 1986; Machado *et al.*, 2012a).

Calving related problems (dystocia, stillbirth, and twins) are also known to increase the risk of uterine diseases (Markusfeld, 1984; Benzaquen *et al.*, 2007; Potter *et al.*, 2010; Cheong *et al.*, 2011), by facilitating the access of bacteria into the uterine mucosa (Bicalho *et al.*, 2010), and by causing uterine tissue damage. Abnormal calving status were more likely to develop metritis (Benzaquen *et al.*, 2007; Giuliodori *et al.*, 2013) and clinical endometritis (Benzaquen *et al.*, 2007) than cows with normal calving. These calving related problems are also independent risk factors for uterine diseases. Independent effects of dystocia, twin parturition, and stillbirth on the incidence of metritis have been reported (Bruun *et al.*, 2002; Bicalho *et al.*, 2010; Dubuc *et al.*, 2010b), and clinical endometritis

(Potter *et al*, 2010; Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). There are studies with conflicting results regarding the association between calving related problems and subclinical endometritis. Cheong *et al*. (2011) reported that these calving abnormalities were associated with subclinical endometritis, whereas others did not observe the same associations (Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). Abortion and induced calving are also factors predisposing to uterine diseases (Kaneene and Miller, 1995; Bruun *et al.*, 2002).

Cows that give birth to males calves are more likely to have uterine contamination after parturition (Bicalho *et al.*, 2010), are more likely to have dystocia (Mee *et al.*, 2011), and stillbirth parturitions (Meyer *et al.*, 2001) than cows having female calves. However, to the best of our knowledge, there is no evidence that having male calves is a direct risk factor for metritis, but it was reported to be a risk factor for clinical endometritis (Potter *et al.*, 2010). The same association was not observed for subclinical endometritis (Cheong *et al.*, 2011).

There is a u-shaped association between parity and metritis; primiparous cows and cows in parity 3 or greater are more likely to develop metritis than cows in parity 2 (Markusfeld, 1984; Saloniemi *et al.*, 1986; Bruun *et al.*, 2002); however, others have not observed this u-shaped association, and simply reported that primiparous cows are more likely to develop metritis than multiparous counterparts (Dubuc *et al.*, 2010b; Machado *et al.*, 2012a). Primiparous are more likely to suffer uterine damage due to dystocia than older cows (Meyer *et al.*, 2001; Uematsu *et al.*, 2013). Similarly to metritis, parity is also a risk factor for endometritis; primiparous cows are more likely to develop clinical or subclinical endometritis than multiparous cows (Dubuc *et al.*, 2010b; Potter *et al.*, 2010; Cheong *et al.*, 2011).

Another cow-related factor that was found to increase the risk of uterine infection was the angle of the vulva (Potter *et al.*, 2010). A vulval angle $<70^{o}$ to the horizontal axis increases the risk of clinical endometritis; this conformation could allow fecal contamination of the vagina, allowing bacteria to access more easily the intrauterine lumen and cause infection.

It has been suggested that there is an involvement of genetic factors in the incidence of metritis, as the heritability of this disease was reported to be as high as 0.19 and 0.26 for primiparous and second lactation cows, respectively (Lin *et al.*, 1989). However, other studies have reported decreased heritability values for metritis, ranging from 0.02 to 0.07 (Lyons *et al.*, 1991; Van Dorp *et al.*, 1998; Zwald *et al.*, 2004a, b). Recent studies have investigated the association between single nucleotide polimorphisms (SNPs) occurring in bovine innate immune genes and uterine diseases (Galvão *et al.*, 2011; Pinedo *et al.*, 2013). Pinedo *et al.* (2013) reported weak associations between metritis, endometritis, and SNPs occurring in genes encoding the toll like receptors 2, 4, 6, and 9. Galvão *et al*. (2011) concluded that uterine health was not affected by the SNP at position +735 in the interleukin-8 receptor-α gene. Polimorphism in the leptin receptor gene was linked with increased metritis incidence (Oikonomou *et al.*, 2009). Although the sire predicted transmitting ability for milk production traits was associated with poorer reproductive performance, it was not linked with increased metritis susceptibility (Bicalho *et al.*, 2014b).

The environment

It is intuitive to think that poor hygiene in the maternity and calving area is a factor predisposing postpartum intrauterine contamination and development of uterine diseases. However, different studies present conflicting data to support its importance. The cleanliness of the perineal region at the time of parturition was associated with metritis (Schuenemann *et al.*, 2011). Herds using straw for calving pen bedding had decreased incidence of metritis (Kaneene and Miller, 1995) and subclinical endometritis (Cheong *et al.*, 2011) than herds using another material; straw could be considered a cleaner bedding material when compared to other materials, such as sand and sawdust. Pasture calvings were also associated with decreased metritis incidence, and the pasture could be also considered as an environment less congested with bacteria than a barn (Kaneene and Miller, 1995). However, other studies have found that poor hygiene is unrelated to uterine diseases; Potter *et al*. (2010) did not observe any association between clinical endometritis and markers of hygiene (fecal consistency score, cow cleanliness score, disinfection of calving equipment, and the wearing of gloves when assisting parturition). Additionally, the microflora of cows from two hygienically contrasting farms was not influenced by the environmental hygiene status; however, these findings should be interpreted with care, because this study was performed in only two herds and enrolled only 26 cows (Noakes *et al.*, 1991).

Individual housing in the maternity facility has been associated with increased risk of metritis (Kaneene and Miller, 1995). Additionally, housing was associated with incidence of subclinical endometritis (Cheong *et al.*, 2011; Prunner *et al.*, 2014). Herds housing early postpartum cows in freestall barns had decreased subclinical endometritis incidence than herds that housed their postpartum cows in bedded packs (Cheong *et al.*, 2011). Prunner *et al*. (2014) reported that tie stall systems were associated with decreased risk of subclinical endometritis when compared with stables with calving pens; however, housing system was not associated with clinical endometritis.

The incidence of metritis has been associated with calving season, but with little agreement on which season is a predisposing factor for uterine diseases (Erb and Martin, 1980; Markusfeld, 1984; Gröhn *et al.*, 1990;

Bruun *et al*., 2002). Markusfeld (1984) reported that cows calving during summer are more likely to be affected with metritis, whereas the incidence of metritis was associated with summer-fall (Erb and Martin, 1980), fall-winter (Gröhn *et al.*, 1990), or winter-spring calvings (Bruun *et al.*, 2002). Heat stress was also reported to be a predisposing factor for RP and consequently metritis (DuBois and Williams, 1980). These discrepancies could be explained by geographical and temporal differences among studies. However, more recent literature reported that season is unimportant for metritis (Dubuc *et al.*, 2010b), and endometritis (Dubuc *et al.*, 2010b; Prunner *et al.*, 2014). Perhaps the advances in management have minimized the detrimental effects of season on postpartum uterine health (Collier *et al.*, 2006).

The pathogens

Virtually all cows will have bacterial contamination in their uterine lumen after parturition (Foldi *et al.*, 2006; Santos and Bicalho, 2012). *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* are considered the primary bacterial causes of uterine diseases (Miller *et al.*, 2007; Bicalho *et al.*, 2010; Santos *et al.*, 2011), but other pathogenic bacteria, such as, *Bacteroides spp*, *Ureaplasma spp*, *Staphylococcus spp*, *Helcococcus spp*, *Prevotella melaninogenicus* and *Streptococcus spp*. have also been associated with uterine diseases (Azawi *et al.*, 2008; Machado *et al.*, 2012c; Locatelli *et al.*, 2013). Although the etiology of uterine diseases is mainly attributed to bacterial infection, the bovine herpesvirus type 4 (BoHV-4) has been associated with poor postpartum uterine health, acting as a secondary pathogenic agent following bacteria (Monge *et al.*, 2006; Donofrio *et al.*, 2009; Chastant-Maillard, 2013).

Escherichia coli

Traditionally, *E. coli* has been described as the main pathogen initiating postpartum uterine infection and disease (Studer and Morrow, 1978; Bonnett *et al.*, 1991; Bicalho *et al.*, 2010; Sheldon *et al.*, 2010). It has been reported that uterine *E. coli* are merely opportunistic environmental bacteria, because none of the virulence factors evaluated in one study were associated with the probability of occurrence of uterine diseases (Silva *et al.*, 2009). Nevertheless, recent studies have characterized important virulence factors that enable *E. coli* to bind and invade the bovine endometrium, making significant advances to understand how *E. coli* plays a role in the pathogenesis of metritis and endometritis (Bicalho *et al.*, 2010; Sheldon *et al.*, 2010).

Silva *et al*. (2009) characterized the phenotype and genotype of 72 *E. coli* isolated from the uterus of metritic and non-metritic cows, and found that none of the 15 virulence factors evaluated were associated with metritis. Sheldon *et al*. (2010) investigated the presence of 17 virulence factors from 114 uterine *E. coli* isolated from 64 postpartum dairy cows and the only virulence factor associated with disease was *fyuA*. However, they found that *E. coli* isolated from cows with metritis were more capable of adhering and invading epithelial and stromal endometrial cells. In a larger scale study, Bicalho *et al*. (2010) explored 32 potential virulence factors, using 611 *E. coli* isolates from 374 cows housed in four different farms in New York State. It was found that six virulence factors common to extra-intestinal and entero-aggregative *E. coli* were associated with uterine diseases: *fimH*, *hlyA*, *cdt*, *kpsMII*, *ibeA*, and *astA*. The virulence factor FimH was the most prevalent and the most important for metritis and endometritis. The FimH protein is an *E. coli* type 1 pili adhesive protein that plays an important role in the adhesion to mannosides (Krogfelt *et al.*, 1990) and enables bacteria to colonize epithelial surfaces (Mooi and de Graaf, 1985). It is known that *E. coli* expressing the type 1 pili containing FimH causes urinary tract infection in humans (Kaper *et al.*, 2004), and it is critical for the ability of these *E. coli* to adhere to and colonize the bladder epithelium (Mulvey, 2002). In fact, it was demonstrated that FimH also mediates adhesion between endometrial pathogenic *E. coli* and the bovine uterine mucosa, because mannose treatment of *E. coli* decreased their ability to adhere to bovine endometrial cells *in vitro* (Sheldon *et al.*, 2010). Recently, an alternative prevention method for metritis using ultrapure mannose was tested, but intrauterine administration of 50 g of mannose in the first three days after parturition was ineffective to reduce bacterial contamination and prevent metritis (Machado *et al.*, 2012a).

It has been suggested that *E. coli* is important for metritis and endometritis in the first week postpartum, especially during the first three days after parturition, potentially inducing changes that will favor subsequent infection by other pathogens (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012). However, its intrauterine presence after the first week postpartum is unimportant for disease and reproductive performance (Bicalho *et al.*, 2012; Machado *et al.*, 2012a, c; Sens and Heuwieser, 2013). Dohmen *et al*. (2000) suggested that the presence of *E. coli* and its endotoxin lipopolysaccharide (LPS) in lochia during the first two days postpartum leads to subsequent *T. pyogenes* infection at 14 days after calving. Similarly, Bicalho *et al*. (2012) found that cows tested positive for the intrauterine presence of the *E. coli* virulence factor FimH at 1-3 DIM were more likely to develop *F. necrophorum* intrauterine contamination at 8-10 DIM. The presence of *E. coli* in the early postpartum period was also associated with impaired reproductive performance (Bicalho *et al.*, 2012; Machado *et al.*, 2012a).

Fusobacterium necrophorum

The combination of anaerobic microorganisms' metabolism and oxygen consumption by PMNs fighting against the intrauterine infection in the first days postpartum decreases the intrauterine oxygen reductase potential, creating an anaerobic environment (El-Azab *et al.*, 1988). This will favor the growth of strict and facultative anaerobes, such as *F. necrophorum* and *T. pyogenes*, respectively. Several studies have identified *F. necrophorum* as an important etiological agent of uterine diseases (Ruder *et al.*, 1981; Noakes *et al.*, 1991; Dohmen *et al.*, 2000). Recent studies using molecular characterization of the intrauterine microbiota have reinforced this assumption. It was reported that *F. necrophorum* was the most prevalent bacteria in samples collected from cows affected with metritis, while being completely absent in samples from healthy cows (Santos *et al.*, 2011). Similarly, it was reported that the intrauterine presence of *F. necrophorum* at 8-10 DIM was associated with metritis (Bicalho *et al.*, 2012), and at 35 days postpartum is associated with clinical endometritis (Machado *et al.*, 2012c).

Fusobacterium necrophorum is a gram-negative, non-spore forming, rod-shaped anaerobe that produces butyric acid as a major product of fermentation (Nagaraja *et al.*, 2005). There are several virulence factors associated with toxicity, adhesion and aggregation that are implicated in the pathogenesis of *F. necrophorum* infections. However, leukotoxin (LKT) is considered the major virulence factor associated with infections in animals (Tan *et al.*, 1994; Narayanan *et al.*, 2002). It is known that LKT is highly toxic to bovine PMNs (Tan *et al.*, 1994), inducing apoptosis-mediated killing of them (Narayanan *et al.*, 2002); this toxicity is dose-dependent (Tan *et al.*, 1992). It is possible than LKT is acting in the uterus by weakening the intrauterine defensive line mediated by PMNs, impairing the ability of the innate immune system to eliminate bacterial infections from the uterus through phagocytosis. Recently, it was reported that the adhesion of *F. necrophorum* to endothelial bovine cells is mediated by outer membrane proteins (Kumar *et al.*, 2013), specifically, the virulence factor FomA (Kumar *et al.*, 2015).

Fusobacterium necrophorum and *T. pyogenes* are known to be synergistic microbes, causing numerous infections in cattle, such as liver, foot, lungs and mandibular abscesses, foot rot, summer mastitis, and calf diphtheria (Nagaraja *et al.*, 2005). This synergy is also observed in uterine diseases (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012; Machado *et al.*, 2012c).

Trueperella pyogenes

Trueperella pyogenes, a Gram positive, non-motile, non-sporeforming, short, rod-shaped bacterium (Jost and Billington, 2005), is a common inhabitant of urogenital, gastrointestinal, and upper respiratory tracts of many animal species (Hagan *et al.*, 1988; Narayanan *et al.*, 1998; Carter and Wise, 2004). However, a physical or microbial insult to the host can lead to a variety of suppurative *T. pyogenes* infections; *T. pyogenes* is an opportunistic pathogen that acts in synergy with *F. necrophorum*, and is consistently associated with metritis and especially endometritis (Studer and Morrow, 1978; Bonnett and Martin, 1995; Williams *et al.*, 2005; Bicalho *et al.*, 2012; Machado *et al.*, 2012a, c).

Trueperlla pyogenes is equipped with several known and putative virulence factors that are important for its pathogenic potential. Its primary virulence factor, pyolysin (PLO), is a potent cholesterol-dependent cytolysin and is associated with the tissue damage caused by *T. pyogenes* infection (Jost and Billington, 2005; Amos *et al.*, 2014). It is known that *T. pyogenes* can provoke a cellular inflammatory response in the uterus, but the intact endometrium is protective against the tissue damage cause by PLO (Miller *et al.*, 2007; Amos *et al.*, 2014). It was demonstrated that the epithelial layer of the endometrium is protective against PLO because epithelial cells contain less cholesterol than stromal cells (Amos *et al.*, 2014). Therefore, it was suggested that *T. pyogenes* acts in the postpartum uterus as an opportunistic pathogen, causing disease once the epithelial layer is lost after parturition, that could have been a result of previous intrauterine infection and/or a traumatic event during parturition, such as dystocia and RP (Dohmen *et al.*, 2000; Bicalho *et al.*, 2012).

Trueperella pyogenes also expresses a number of surface-exposed proteins, such as fimbriae, neuraminidases, and extracellular matrix-binding proteins, which are involved in adherence and mucosal colonization (Jost and Billington, 2005; Pietrocola *et al.*, 2007; Santos *et al.*, 2010; Machado and Bicalho, 2014). Although there were no associations between virulence factors and uterine diseases in one study (Silva *et al.*, 2008), others reported that virulence factor encoded by the gene *fimA* was associated with metritis (Santos *et al.*, 2010) and clinical endometritis (Bicalho *et al.*, 2012).

Other pathogens

A wide variety of other bacteria has been associated with postpartum uterine health of dairy cows. However, there are no details on their roles on the pathogenesis of metritis and endometritis. It was reported that *Bacteroides spp.* contributes to clinical endometritis, acting in synergy with *T. pyogenes* and *F. necrophorum* (Dohmen *et al.*, 1995; Machado *et al.*, 2012c). *Prevotella melaninogenica* was consistently isolated from diseased bovine uterus (Olson *et al.*, 1984), and its intrauterine relative abundance in the 7th week postpartum was increased for cows affected with clinical endometritis (Machado *et al.*, 2012c).

Non-hemolytic *Streptococcus spp.* and *Mannheimia haemolytica* were associated with the fetid mucus odor, a characteristic sign of uterine infection (Williams *et al.*, 2005). The intrauterine presence of *Streptococcus uberis* on the third day of lactation was reported to be highly associated with the risk of clinical endometritis (Wagener *et al.*, 2014). By the use of a metagenomic technique, *Helcococcus spp* was described to be associated with clinical endometritis (Machado *et al.*, 2012c); *Helcococcus kunzii* and *Helcococcus ovis* were isolated from metritic uterus of dairy cows (Locatelli *et al.*, 2013), suggesting that these species may play a role in the pathogenesis of uterine diseases. Furthermore, *Ureaplasma spp* was highly prevalent in the uterus of cows affected with clinical endometritis (Machado *et al.*, 2012c); *Ureaplasma diversum* has been associated with granular vulvitis, endometritis and reproductive failure (Doig *et al.*, 1980; Kreplin *et al.*, 1987). *Staphylococcus spp.* is another bacterium that has been previously associated with poor uterine health and impaired reproduction (Paisley *et al.*, 1986; Machado *et al.*, 2012c).

The BoHV-4 is the only virus that has been consistently associated with uterine infection of dairy cows (Parks and Kendrick, 1973; Monge *et al.*, 2006; Donofrio *et al.*, 2009, 2010; Chastant-Maillard, 2013; Jacca *et al.*, 2013). It was described that BoHV-4 can cause latent infection in bovine macrophages (Donofrio and van Santen, 2001), and are tropic for bovine endometrial epithelial and stromal cells, replicating and leading to non-apoptotic cell death (Donofrio *et al.*, 2007; Jacca *et al.*, 2013). The endometrium can respond to the BoHV-4 presence with an inflammatory response, overexpressing pro-inflammatory cytokines IL-8 and TNF-α (Donofrio *et al.*, 2010; Jacca *et al.*, 2013). It has been suggested that BoHV-4 acts in cooperation with bacterial infection to cause disease in the uterus of dairy cows (Donofrio *et al.*, 2008).

Conclusion

Metritis and endometritis are highly prevalent in postpartum dairy cows and both diseases have a negative impact in the modern dairy enterprise. They are complex multifactorial diseases, and a wide range of factors contributes to their occurrence. They are often associated with mixed bacterial infection of the uterus, and the major pathogens associated with uterine diseases are *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* These infections are more likely to develop under some conditions related the host and to the environment. Environmental factors that can predispose metritis and endometritis are poor hygiene at calving, housing type and calving season. Events during the transition period related to negative energy balance and metabolic imbalance, mineral deficiencies, leading to immunosuppression are also of great important during establishment of intrauterine bacterial infections. This, combined with endometrium trauma events during parturition, such as calving related problems, increases the risk of metritis and endometritis. To understand all these factors, and their relationship and interactions, is key to implementing management practices to mitigate the risk of disease, and to develop new strategies to treat and prevent metritis and endometritis. Recently, encouraging preliminary results regarding the effectiveness of multivalent vaccines containing components of *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum* were published (Machado *et al.*, 2014b). It was reported that boosting the host immune system by systemically immunizing late pregnant heifers against cellular components and important virulence factors of these pathogens reduced the incidence of puerperal metritis. However, more research is needed to advance the knowledge on the pathogenesis of uterine diseases, and to develop better strategies to ameliorate immunosuppression during the transition period of dairy cows.

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In vitro production of bovine embryos: revisiting oocyte development and application of systems biology

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Abstract

In vitro production (IVP) of bovine embryos has become a widespread technology implemented in cattle breeding and production. The implementation of genomic selection and systems biology adds great dimensions to the impact of bovine IVP. The physical procedures included in the IVP process can still be improved, and aspects related to the oocyte donor, oocyte maturation and the recipients are addressed in the following. Also, some of the future aspects of genomic selection and systems biology are addressed with particular focus on the Brazilian-Danish collaboration in the so-called GIFT-project.

Keywords: cattle, embryo, fertilization, genome, oocyte.

Introduction

Over the past years, *in vitro* production (IVP) of bovine embryos has become implemented in cattle breeding in many countries. Particularly South and North America are regions where the technology has gained great impact. From a biological perspective, IVP has circumvented some of the early drawbacks related to serum-rich embryo culture conditions resulting in the large offspring syndrome (LOS). From a breeding perspective, the implementation of ultrasound-guided ovum pickup (OPU), quantitative genomics and systems biology has opened new possibilities for selection of oocyte donors and embryos for improved breeding strategies.

In spite of the improvements of the *in vitro* procedures for mimicking oocyte maturation, fertilization and initial embryonic development, IVP still needs refinements. The present review aims at revisiting, in particular, oocyte development, to pin point aspects where the bovine IVP systems are still suboptimal as compared with *in vivo* development, as well as aspects related to oocyte donors differing in reproductive stage and age. Also, we aim at pointing towards implementation of systems biology in bovine IVP and giving some practical considerations on the implementation of that technology in Denmark; a country where cattle breeding has reached a high level of perfection, but the implementation of bovine IVP has been delayed due to ethical animal welfare considerations related to OPU and LOS.

Revisiting oocyte development

Oocyte development in postpubertal heifers and cows

The development of a competent oocyte to be present for fertilization in the oviduct can roughly be divided into three distinct phases. The basic cell structure (ultrastructure) and developmental competence of the oocyte is generated during the first phase (the oocyte growth phase), when oocyte growth accompanies follicular growth from the primordial to the small (2-3 mm) tertiary (antral) follicle. During the antral phase when follicles in a cohort reach a diameter of about 3-5 mm, one dominant follicle is selected, as opposed to a group of subordinate follicles (Fig. 1; Ginther *et al.*, 1989). The ultrastructure of the oocyte in the dominant follicle enters a second phase (oocyte prematuration or capacitation), where it is modified reflecting an increase in oocyte developmental competence. In the ovulatory follicle, i.e. the dominant follicle of the last follicular wave, the oocyte undergoes a third and last phase of ultrastructural changes (oocyte maturation) during an approximately 24 h period between the peak of the LH-surge and ovulation.

When oocytes are aspirated for IVP from follicles of 2-8 mm in diameter, they are harvested from a heterogeneous and not fully competent pool of follicles. Hence, they originate from antral follicles of non-ovulatory or ovulatory follicular waves and from early dominant or early subordinate follicles. Consequently, the oocytes have not completed the acquisition of developmental competence, which was projected in the dominant follicle (oocyte prematuation), and some of them may even have encountered the environment of initial atretic follicles.

In order to reveal the ultrastructural changes during the three phases of oocyte development, we carefully processed oocytes for transmission electron microscopy from preantral follicles (primordial, primary and secondary follicles; (Fair *et al*., 1997) as well as from antral follicles, with appreciation of their status as

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being dominant or subordinate (Assey *et al*., 1994), and from preovulatory follicles approaching ovulation (Hyttel *et al*., 1986). The exact definition of dominant *vs*. subordinate follicles was achieved by administration of prostaglandin-F2alpha at day 7 after ovulation. This treatment stimulates ovulation of the dominant follicle of the first follicular wave. This simple model allows for harvesting of oocytes from well-defined stages of follicular dominance and subordination. The ultrastructural changes associated with oocyte development over the three phases of follicular development are presented in Fig. 2.

From the data presented in Fig. 2, it is obvious that the oocyte during the growth and prematuration phases undergoes marked changes that are reflected in its developmental competence and which are significant for obtaining full oocyte competence after maturation *in vivo* as well as *in vitro*. According to functional studies of bovine oocytes at different stages of their development, it appears that the oocyte achieves its basic competence for completing meiotic maturation to meiotic metaphase II (MII) at a diameter of around 110 µm (i.e. during the oocyte growth phase at the time when transcription decreases), and at a diameter of about 120 µm it attains the competence for subsequent blastocyst development. Hence, in oocytes harvested from follicles less than 3 mm in diameter, the oocyte has not completed the growth phase and build up the basic requirement of mRNA and proteins to sustain development (Fair *et al*., 1995, 1996, 1997). During the subsequent prematuration phase, the oocyte exhibits further structural modulations (Fig. 2) and its developmental competence increases (Assey *et al*., 1994). Strikingly, the oocytes of the subordinate follicles, which are entering into the early phase of atresia, may display very similar changes as seen on the oocyte of the dominant follicle. It will under normal circumstances be oocytes from follicles undergoing prematuration or initial atresia, which will be aspirated for bovine embryo IVP.

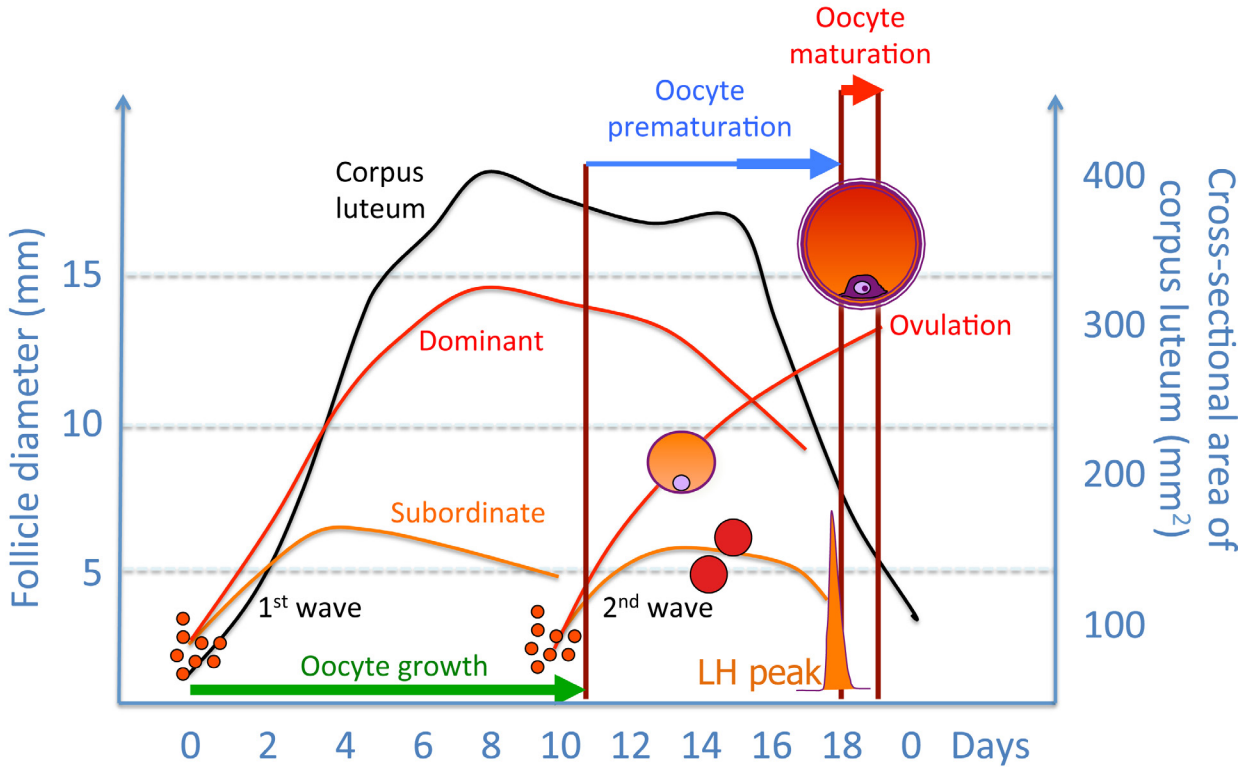

Figure 1. Follicular waves and phases of oocyte development in cattle (adapted from Ginther, 1998). The lower axis indicates days after ovulation.

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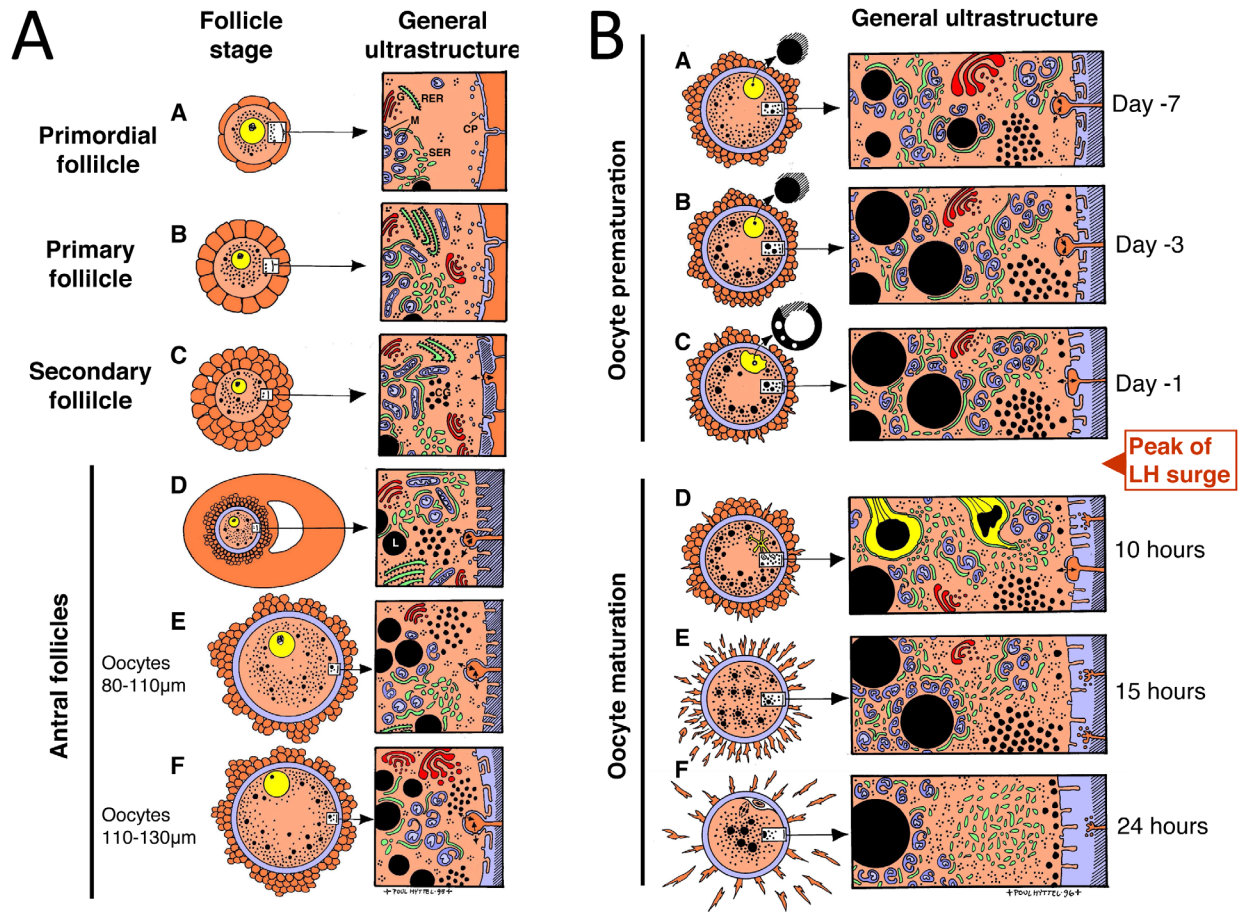

Figure 2. **(A)** Schematic drawing of bovine oocyte growth. **(A_A)** Primordial follicle with oocyte surrounded by a single layer of flattened granulosa cells. The central oocyte nucleus (yellow) is surrounded by round mitochondria (M), smooth (SER) and rough (RER) endoplasmic reticulum and small Golgi complexes (G). The oocyte cortex presents numerous coated pits (CP) and vesicles. The oocyte is transcriptionally quiescent. **(A_B)** Primary follicle with oocyte surrounded by a single layer of cuboidal granulosa cells. The eccentric oocyte nucleus is surrounded by round and elongated mitochondria. **(A_C)** Secondary follicle with oocyte surrounded by more than one layer of cuboidal granulosa cells. Small patches of zona pellucida material (hatched areas) have appeared and gap junctions (small arrows) developed between the oocyte and the granulosa cells. In the oocyte, the first small clusters of cortical granules (CG). The oocyte displays initial transcriptional activity. **(A_D)** Early tertiary follicle up to about 1mm. The follicular antrum has developed and the oocyte is surrounded by cumulus cells of which the innermost possess projections that penetrate the zona pellucida, invaginate the oolemma and make gap junctional contact to it. In the oocyte, the organelles have attained a more even distribution throughout the ooplasm, elongated mitochondria have become more numerous, lipid droplets (L) have become common, and the number and size of the cortical granule clusters have increased. Erect microvilli have become embedded within the zona pellucida. The oocyte is transcriptional active. **(A_E)** Tertiary follicle up to about 3 mm as represented by oocytes at 80 to 110 µm in diameter. The number of lipid droplets in the oocyte has increased. Oocytes less than 100 µm are transcriptionally active, whereas such at 100 to 110 µm transcription decreases in abundance. **(A_F)** Larger tertiary follicles as represented by oocytes at more than 110 µm in diameter. In the oocyte, the organelles have been dislocated to the periphery, the number of lipid droplets have increased as have the size of the Golgi complexes. The microvilli have been released from the zona pellucida and pile up in stacks in the perivitelline space. The peripheral oocyte nucleus presents has deceased its transcriptional activity to a minimum. **(B)** Schematic drawing of ultrastructural aspects of bovine oocyte prematuration in the dominant follicle up to the LH peak and maturation after the peak. **(B_A)** Oocyte from a dominant follicle 6 days before the LH peak. The general ultrastructure is identical with that obtained at the end of oocyte growth (A_F). **(B_B)** Oocyte from a dominant follicle 3 days before the LH peak. The number of microvilli stacks have decreased as have the size of the Golgi complexes, the amount of lipid droplets has increased, and the cortical granule clusters have dislocated to a more superficial location. **(B_C)** Oocyte from a dominant follicle on the day before the LH peak. Some individual corona cells display elongation and the corona cell projections have been retracted to a more superficial location, the perivitelline space has enlarged, the microvilli have become more erect, and the size of the Golgi complexes has been further reduced. Moreover, the envelope of the oocyte nucleus has become undulating and the nucleolar remnant has transformed into a ring-like structure. **(B_D)** Oocyte at "germinal vesicle breakdown" from an ovulatory follicle at 9-12 h after the LH peak. The perivitelline space develops further and in the oocyte the mitochondria tend to arrange around the lipid droplets and the nuclear envelope is dissolved into tubules of SER and microtubules appear adjacent to the condensing chromosomes. **(B_E)** Oocyte at MI from an ovulatory follicle at about 15 h after the LH peak. The number and size of the lipid droplets has increased and mitochondria have assembled around the droplets and these conglomerates have attained a more even distribution. Numerous ribosomes have appeared especially around the chromosomes and the size of the Golgi complexes has decreased further. **(B_F)** Oocyte at MII from an ovulatory follicle at about 24 h after the LH peak. The bulk of the cortical granules are distributed at solitary positions along the oolemma. The lipid droplets and mitochondria have attained a more central location in the ooplasm leaving a rather organelle free peripheral zone in which the most prominent features are large clusters of SER (adapted from Hyttel, 2011).

Age and status of the oocyte donor

There is an increasing trend within cattle breeding to apply OPU to younger and younger females and subsequently IVP in order to accelerate the genetic progress by reducing the generation interval. Also, some donors are in high lactation and/or in early pregnancy. Each of these different situations presents a particular challenge with respect to oocyte developmental competence, which can change according to status of the donor.

Ovaries in young females contain preantral and antral follicles, with the total number of follicles varying between individuals (Silva-Santos *et al*., 2013). The number of growing follicles rises rapidly between 50-80 days postnatally and increases up to 120 days. This follicle growth is stimulated by a transient increase in FSH and LH secretion, which later decreases until immediately prior to the first ovulation, at which time LH serum concentration and pulsatile secretory profile increase and change. During the prepubertal period, follicular waves are present and are preceded by FSH peaks (Erickson, 1966; Rawlings *et al*., 2003). Even though it is possible to aspirate antral follicles from very young heifers, the oocytes will have a decreased developmental competence compared to adults (Steeves *et al*., 1999), illustrated by differences in e.g. ultrastructure (Duby *et al*., 1996), oocyte metabolism (Steeves and Gardner, 1999), and cytoplasmic maturation (Salamone *et al*., 2001).

In high-yielding postpartum cows, low concentrations of circulating steroids have been measured, which could indicate that the dominant follicles are producing less oestrogen affecting the follicle growth phase (Lopez *et al*., 2004; Sartori *et al*., 2004). Furthermore, the negative energy balance in postpartum dairy cows has an adverse effect on oocyte quality, due to the changed endocrine and metabolic profiles (Leroy *et al*., 2008). In pregnant animals, follicular growth is possibly affected by elevated levels of progesterone (Adams *et al*., 1992; Dominguez, 1995).

As illustrated above, the expectations and results from OPU and IVP in cattle depend on the reproductive and physiological status of the oocyte donor, determined by factors such as genetics, age, breed, nutrition, pregnancy, milk yield etc. More research is needed to investigate the possibilities for evaluation and improvement of oocyte quality by e.g. hormone treatments based on the individual status of the donor (e.g. Ireland *et al*., 2007). Furthermore including a period of FSH withdrawal before aspiration has demonstrated positive effects on IVP results ("coasting"; Blondin *et al*., 2012; Nivet *et al*., 2012). Finally, the possibilities for using plasma anti-mullerian hormone (AMH) as an estimator of donor potential should be further investigated including young females (Silva-Santos *et al*., 2013; Guerreiro *et al*., 2014).

Novel developments in media for *in vitro* production

For many years, home-made media compositions based on commercially available stock solutions have been used for bovine *in vitro* embryo production; Tissue Culture Medium 199 (TCM 199; Sigma-Aldrich), Tyrode's Albumin Lactate Pyruvate (TALP) stocks (Parrish *et al*., 1986) and Synthetic Oviduct Fluid (SOF) with few modifications (Tervit *et al*., 1972; Holm *et al*., 1999) and most of them containing serum. All media for IVF are based on a balanced salt solution, amino acid solutions and pyruvate. Further supplementations are vitamins, EDTA, and metal ion buffers.

With the increasing implementation of IVP of bovine embryos worldwide for commercial use, there is an increased focus on optimizing the yield of blastocysts. Furthermore, increased focus on regulatory restrictions on import/export of embryos cultured in media containing serum due to the risk of spreading pathogens, has increased the wish to supplement the IVP media with bovine serum albumin (BSA) and synthetic serum replacements, instead of serum. Currently a Danish company, EmbryoTrans Biotech ApS, is developing a novel culture medium without any animal originating protein source and strictly synthetic serum based.

As medium for *in vitro* culture (IVC) of the embryos, SOF has been used as a continuous culture medium system. However, an increasing interest at some commercial bovine laboratories to perform sequential culture has evolved. The sequential culture system has been widely used for years in human *in vitro* fertilization (IVF), based on the theory that the embryo has different needs depending on the developmental stage. Hence, the media are composed to provide the optimal support from embryo cleavage stage to the blastocyst stage development (Simon, 2002). However, in human IVF the monoculture medium system is gaining popularity again. The monoculture medium is supplemented with all the required compounds to sustain embryo development to the blastocyst stage, and is based on letting the embryo choose the nutrients and components needed for an optimum development during the entire culture period (Gardner *et al*., 2002). It has been suggested that monoculture medium system is as efficient as the sequential medium system (Macklon *et al*., 2002). Knowing that the embryos worst enemy is the fluctuations, in particularly, of pH and temperature (Swain, 2010), a monoculture medium system has the advantage of decreasing the number of manipulations and the length of time the embryo is out of the incubator. The early embryo produces autocrine/paracrine factors, essential for *in vitro* survival (Gopichandran and Leese, 2006), thus, a monoculture medium system may well be the preferred solution.

With the increasing production of bovine IVP

embryos commercial media are becoming available. The developmental rates and gene expression of IVP blastocysts are affected by the use of different IVP media systems. IVP methods have been evaluated by assessing the health of the offspring born (Wrenzycki *et al*., 2004; Bonilla *et al*., 2014), and recent research has focused on finding a new method, where the quality of the embryo and subsequent calf produced in a certain IVP system, can be evaluated before transfer to the recipient. This research has been centered around finding differences in gene expression and epigenetic modifications between *in vivo* and *in vitro* produced embryos, and a long list of candidate genes, believed to be involved in the critical processes of embryo development, is now available (Wrenzycki *et al*., 2004, 2005; Thompson *et al*., 2007; Wrenzycki *et al*., 2007; Chen *et al*., 2013). Therefore, more studies should be conducted to investigate the correlation to healthy live born offspring from embryo quality in terms of media influence on embryo development such as: gene expression, morphology, kinetics and general blastocyst rates. As abundance of gene expression in itself is not a quality marker the studies should be performed including comparison of *in vivo* produced embryos.

Recently, a preliminary study was published showing increased blastocyst rates, superior embryo quality, and more abundant gene expression in embryos produced in the media system from the Danish company EmbryoTrans Biotech compared to the IVP media system from Minitube Germany (Nielsen *et al*., 2014). The selected genes for their proposed value as quality markers for IVP of bovine embryos that were included in the analysis were: Stress response: HSPA1A (heat shock protein), Glucose transport: SLC2A1 and SLC2A3, DNA methylation: DNMT3A (DNA methyltransferase), Maternal recognition of pregnancy: IFNT2 (Interferon tau), Insulin-like growth factor system (growth): IGF1R, Apoptosis: BAX and BCL1L (pro- and antiapoptotic), G6PD (glucose metabolism) and FASN (fat metabolism).

At oocyte aspiration, by e.g. OPU, the oocytes recovered from the same ovary will be at different stages of prematuration or early atresia.. One approach to deal with this situation has been to induce a temporary arrest of oocyte maturation, where work was done a decade ago (Lonergan *et al*., 2003; Donnay *et al*., 2004; Vigneron *et al*., 2004). Recently, this concept was launched again through a specially designed medium, referred to as simulated physiological oocyte maturation (Albuz *et al*., 2010). The results have been varying, and a modified second version is now being tested (Gilchrist *et al*., 2015), illustrating that a practical solution to such a complex challenge is not always so easy. Likewise, questions remain with respect to how the oocytes from very young heifers (older calves) should potentially be treated for optimal results.

Finally, there are many techniques to assess embryonic quality, which include hatching rate analysis, survival to cryopreservation, cell counts of the inner cell mass and trophectoderm, measurement of apoptotic cells and analysis of incidence of chromosomal anomalies (Munoz *et al*., 2014). However, the above procedures have limited practical and wide-spread application for ET in farms. It would be worthwhile to evaluate the pre-implantation embryos for their genetic merit for economic traits and use them in genomic selection – a method that evaluates an animal before it is born. In the accompanying paper by Kadarmideen *et al*. (2015), we describe how genomic screens of pre-implantation embryos could accelerate genetic improvement.

Multi-omics data and application of systems biology to bovine IVP

Animal and veterinary bio-sciences are going through paradigm shift from single low-throughput experiments generating single-layer biological data to often a single integrated experiment, where multi–omics biological data are being generated on individual animals. Modern high-throughput technologies generate data at all levels of the animal biological systems (e.g. genome-wide, transcriptome-wide, metabolome-wide or proteome-wide measurements). This includes reproductive systems and various cell types including ovaries, oocytes, embryos and endometrium. Another angle to -omics data is the emergence of "phenomics", which refers to tens of thousands of phenotypes observed in a single animal instead of a few dozens or a hundred.

Systems biology (SB) approaches, by necessity, involve systematic data collected at all levels of the biological systems and at the individual (animal level) and are aimed at studying interactions between all these levels, but not at one level in isolation, (Kadarmideen, 2008, 2014). It attempts to provide a holistic view of the entire outcome. If reproductive success is an outcome, then it tries to provide an experimental data-driven and hypothesis-based explanation for reproductive success. To achieve this, systems biology collectively models and analyzes these multi-omics datasets using a combination of mathematical, computational biology and bioinformatic principles and tools (Kadarmideen, 2008, 2014). SB is a discipline that iterates between data-driven and hypothesis-driven approaches to understand the whole biological system and provide a complete blueprint of functions of phenotype or a complex disease evolution. Therefore, it requires multi-disciplinary expertise in one team, from mathematical sciences through quantitative genetics to molecular biology.

The term "Systems Genetics", a branch of SB was originally proposed by Kadarmideen *et al*. (2006) which integrate 'omics scale measurements from genome to metabolome to functome through transcriptome and proteome. This systems genetics approach has been applied in livestock (Kadarmideen

and Janss, 2007; Kogelman *et al*., 2014), humans (Civelek and Lusis, 2014) and has been thoroughly reviewed (Li, 2013; Kadarmideen, 2014). One such way is the integration of genomics and transcriptomics by detecting expression QTLs (eQTLs). An eQTL is a genomic region associated with transcript levels, which subsequently affects the phenotype. Systems genetics has been shown to be a powerful method to find important causal and regulatory genes and their variants in predicting biomarkers (for instance reproductive success via a conventional artificial insemination (AI) or embryo transfer (ET).

Several genomic or transcriptomic studies, in isolation, have been conducted to reveal genetic architecture or gene regulatory mechanisms underlying phenotypes or mechanisms that determine pregnancy in bovines, based on transfer of both *in vivo* and *in vitro* produced embryos. There are some large-scale transcriptomic studies in understanding conceptus-maternal communication, which is vital for the establishment and maintenance of pregnancy.

For instance, Bauersach *et al*. (2006) showed that expression of AGRN, LGALS3BP, LGALS9, USP18, PARP12 and BST2 in the endometrium plays a central role in the context of early embryo-maternal communication and pregnancy. Clemente *et al*. (2011) showed differentially expressed genes between day 7 and 13 embryos derived *in vivo* or *in vitro* showing the top 40 up- and down regulated genes on day 13 unique to *in vivo* embryos, unique to *in vitro* embryos, and common to both. (Mamo *et al*., 2011), using RNA Seq technology, generated transcriptomic profiles of bovine conceptuses across the entire pre- and peri-implantation periods (day 7, 10, 13, 16 and 19) and identified clusters of genes associated with blastocyst formation, conceptus elongation, maternal recognition of pregnancy and initiation of implantation. Mamo *et al*. (2011) studied the transcriptome of the uterine endometrium to unravel the genes and pathways governing growth and development of the cattle conceptus. They focused particularly on the time of hatching of the blastocyst from the zona pellucida and its subsequent elongation coincident with the time of maternal recognition of pregnancy.

However, the SB or systems genetics approaches, as described above, for studies of conceptus-maternal communication encompassing multi-omics datasets are lacking. This is exactly one of the focal areas of the Danish-Brazilian GIFT consortium activities (www.gift.ku.dk). As one aspect, the GIFT project envisions to apply transcriptomics and eventually systems genetics approaches to follicular cells to characterize donor cow transcriptomics and systems biology. Briefly, this involves aspiration of oocytes from slaughtered donor cows with production data and subsequent collection and extraction of RNA from mural and cumulus granulosa cells for the RNA seq analysis. Oocytes *in vitro* matured and fertilized with semen from high genetic and low genetic merit bulls are then used to study the effect of genomics-by-sire interaction on blastocyst rates. Furthermore correlation between gene expression in granulosa cells and blastocyst rate, values retrieved for each animal, is analyzed implementing statistical linear models. The analyses will identify differentially expressed genes that can be potential markers for the characterization of donor cows for IVP procedures.

Conclusions and perspectives

The past decade has brought an impressing amount of new knowledge related to bovine OPU-IVP and ET at the biological, physiological, molecular, genetically and especially the practical level. The overall process is basically fairly simple and straightforward, but with the growing knowledge there will be more and more options for making selections along the process. This would be valuable as new traits used to improve overall fertility from a breeding point of view. In addition it also provides an incoming "personalized approach" in bovine assisted reproductive technologies (ARTs) with e.g. individual treatment of the donor based on her status and actual situation; of the bull based on his sperm's reaction to the treatment prior to IVF and of the recipient endometrial status to secure the implantation of the IVP embryo.

Future reflections should take into consideration new traits as decision support tools for reproductive biotechnologies, such as selection of donor cows on embryo production in terms of ability to perform in the IVP laboratory. Quantitative traits such as number of oocytes and number of embryos, and qualitative traits such as quality of oocytes, cleavage rate, development rate, morphology and kinetics of the resultant embryos, are important traits to identify in the fast progressing era of bovine IVP.

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State of the art of GnRH - based timed AI in beef cattle

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Abstract

Based upon observations across a series of experiments that pregnancy rate to timed-AI was positively related to length of proestrus, the traditional 7-day CO-Synch + CIDR program was modified to allow an increased interval from PGF/CIDR removal to GnRH/timed-AI; resulting in the 5-day CO-Synch + CIDR program. This modification has been demonstrated to increase timed-AI pregnancy rates relative to the traditional approach. The impact of this modification on preovulatory estradiol concentrations, as a result of extending the period of gonadotropic stimulus provided to the follicle, initiation of proestrus at a time when ovulatory follicles are highly estrogenic and/or through reduction in the incidence of ovulation of very young follicles, are potential mechanisms for increased estradiol concentrations and enhanced fertility. Conversely, for females in these estrous control programs in which follicular growth is adequately controlled, differences in age of the ovulatory follicle may not be a significant contributor to variation in timed-AI pregnancy rate.

Keywords: bovine, estradiol, fixed-time artificial insemination.

Introduction

The most commonly used approaches to timed-AI in beef cattle in the USA are based upon the CO-Synch program (Geary and Whittier, 1998). In the USA, three hormones are available to synchronize cows; progesterone (usually a vaginal insert; CIDR), prostaglandin F2α (PGF; or it's analog) and GnRH. The original CO-Synch program consisted of an initial GnRH treatment, PGF 7 days later to induce luteolysis, and a second GnRH treatment 48 h after PGF to induce ovulation for timed-AI. The timing of the second injection of GnRH determines the length of "proestrus", or the interval between the initiation of regression of the corpus luteum (CL) and the LH surge. A CIDR is usually inserted into beef females between the initial GnRH and the PGF treatment, resulting in a CO-Synch + CIDR program. Each exogenous hormone used in this program has specific actions and the efficacy and accuracy of these actions are crucial for synchronization. The first GnRH treatment is used to induce ovulation and reset follicular growth. In other words, approximately 1 to 2 days after GnRH a new follicular wave should be initiated in a majority of cows (Thatcher *et al*., 1989; Macmillan and Thatcher, 1991; Twagiramungu *et al*., 1994, 1995). The efficacy of the initial GnRH, however, varies among animal class and stage of the estrous cycle (Pursley *et al*., 1995; Geary *et al*., 2000; Atkins *et al*., 2008, 2010; Souza *et al*., 2009). The second GnRH will induce an LH surge and subsequent ovulation of the dominant follicle that results from the new wave induced by the first GnRH. Luteolysis is induced with PGF between 48 and 72 h before the second GnRH treatment. Timed-AI is performed coincident with the second administration of GnRH. One important concern is the proportion of cows that are induced to ovulate follicles that are smaller than typical diameter with the second GnRH administration and the fact these animals are less likely to become pregnant to timed-AI (Lamb *et al*., 2001; Perry *et al*., 2005).

The influence of ovulatory follicle maturity on fertility in beef cattle has been investigated (Perry *et al*., 2005; Mussard *et al*., 2003, 2007; Bridges *et al*., 2010). One hypothesis was that diameter of ovulatory follicles was the most appropriate indicator of follicle "maturity" and that cows induced to ovulate small follicles would have lesser fertility compared to those induced to ovulate larger follicles. Within each of three experiments (Table 1; Mussard *et al*., 2003, 2007) this hypothesis was supported, but as data from multiple experiments accumulated, the relationship of follicle diameter to pregnancy rate appeared inconsistent. Across experiments, the more consistent predictor of pregnancy rate appeared to be duration of proestrus (interval from initiation of CL regression with PGF to the LH surge; Table 1). Based on the relationship between length of proestrus and conception rate, an additional experiment (Table 1; Bridges *et al*., 2010) was performed to hold follicle diameter constant and only vary length of proestrus. It was demonstrated that at a constant ovulatory follicle diameter, length of proestrus had a substantial influence on conception rate. Taken together, data from this series of studies suggested a strong positive relationship of duration of proestrus with follicle maturity and fertility and suggested that diameter of the ovulatory follicle, in itself, was not a consistent predictor of follicle maturity. The effect of ovulatory follicle diameter at GnRH-induced ovulation or at spontaneous ovulation on conception rate has also been evaluated (Perry *et al*.,

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2005, 2007). It was reported that diameter of the ovulatory follicle influenced conception rate after detection of estrus in heifers, but not in postpartum cows. In postpartum cows that did not exhibit estrus, diameter of the ovulatory follicle was positively associated with conception rate when ovulation was induced with GnRH. Thus, if a 'complete' spontaneous proestrus occurred in cows (confirmed by exhibition of estrus), diameter did not impact fertility, but, diameter of the ovulatory follicle when ovulation was induced with GnRH did influence conception rate; at a constant duration of proestrus. Since findings suggested that maturity of the ovulatory follicle and probability of conception was perhaps defined by length of proestrus, we applied this knowledge towards optimizing the existing CO-Synch + CIDR program.

Table 1. Conception rate, diameter and age of the ovulatory follicle, length of proestrus, and number of cows in a series of experiments investigating the effect of follicle maturity on fertility.

Conception rate (%)[a]	Follicle diameter at ovulation (mm)[b]	Duration of proestrus (days)[c]	n	Experiment
4	11.1 ± 0.2	1.0 ± 0.1	45	Mussard *et al*., 2003a[e]
8	11.1 ± 0.2	1.0 ± 0.1	12	Mussard *et al*., 2003b[f]
10	12.6 ± 0.2	1.25	10	Bridges *et al*., 2010[g]
57	13.6 ± 0.2	2.2 ± 0.1	54	Mussard *et al*., 2003a[e]
67	13.7 ± 0.2	2.0 ± 0.1	12	Mussard *et al*., 2003b[f]
71	12.9 ± 0.2	2.25	28	Bridges *et al*., 2010[g]
76	10.7 ± 0.1	3.3 ± 0.1	29	Mussard *et al*., 2007[d]
100	12.0 ± 0.3	4.7 ± 0.2	24	Mussard *et al*., 2007[d]

[a]Percentage of animals determined to be pregnant following insemination. Pregnancy determination was conducted via ultrasonography at approximately 30 days post-insemination. [b]Diameter of the largest ovulatory follicle as determined by ultrasonography conducted either at GnRH administration or estrus. [c]Interval from PGF2α until GnRH administration. [d]Cows were either induced with GnRH to ovulate a small (~11 mm) follicle or allowed to spontaneously exhibit estrus. Cows were inseminated 12 h following estrus or GnRH.[e]Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Animals were inseminated 12 h following GnRH administration. [f]Cows were induced to ovulate either a small (~11 mm) or large (~13 mm) ovarian follicle with GnRH. Embryo from non-treated cows were then transferred 7 days after GnRH. [g]Cows were induced to ovulate an ovarian follicle of similar diameter with GnRH either 1.25 or 2.25 days following PGF2α administration. Animals were inseminated 12 h following GnRH administration. Includes only cows with a luteal phase of normal length.

Lengthening proestrus in the CO-Synch + CIDR program

The length of proestrus with the traditional 7-day CO-Synch + CIDR program was varied from 50 to 66 h in mature cows without influencing timed-AI pregnancy rate, but in younger cows (≤3 years of age), greatest pregnancy rates were achieved with timed-AI at 56 h as compared to longer intervals (Dobbins *et al*., 2009). Others (Busch *et al*., 2008) have reported that timed-AI pregnancy rates were greater when proestrus was 66 than 54 h. In practice, the second GnRH is given and timed-AI is performed in most herds between 54 and 66 h after PGF. We hypothesized that if the CO-Synch + CIDR synchronization approach could be modified in a manner in which we could increase the interval from PGF and CIDR removal to the second GnRH and timed-AI, that timed-AI pregnancy rate would increase. This end was achieved through development of the 5-day CO-Synch + CIDR program (Bridges *et al*., 2008). This paper will focus on the physiological effects of this change in the program and potential mechanisms for the increase in timed-AI pregnancy rate that is achieved.

Hormonal changes with a lengthened proestrus

Proestrus starts with removal of progesterone sources (a CL, a CIDR or both) and ends with either a spontaneous or GnRH- induced LH surge. Concentrations of progesterone decline rapidly and are sustained at basal concentrations throughout proestrus, setting off a series of crucial hormonal changes that precede ovulation. An almost immediate response to declining progesterone concentrations is an increase in the frequency of LH pulses. Frequency of release of LH from the anterior pituitary is primarily regulated by progesterone and the negative association of progesterone concentration and frequency of LH pulses has been well established (Kinder *et al*., 1996). Proestrus is characterized by LH pulses at an increasing frequency as proestrus progresses and the LH surge approaches (Imakawa *et al*., 1986). Pulsatile LH secretion is the primary factor that drives the final development of preovulatory follicles. During a spontaneous proestrus, growth of the preovulatory follicle and production of estradiol by granulosa cells in the follicle increases as proestrus progresses. We have compared preovulatory estradiol and post-ovulatory progesterone concentrations, and the magnitude of the

LH surge between female cattle experiencing either a long (54 h; LPE) or short (30 h; SPE) proestrus after synchronizing follicular growth with ultrasound-guided aspiration of the dominant follicle and altering timing of luteal regression (Bridges *et al*., 2010). Ovulatory follicle size and magnitude of the GnRH-induced LH surge did not differ, but there tended to be a greater incidence of short estrous cycles and lesser progesterone concentrations during the subsequent estrous cycle in the SPE than LPE treatment. The most striking difference between treatments was that concentrations of estradiol were greater in the LPE than SPE treatment during the 38 h preceding GnRH (Fig. 1). Consistent with this observation, cows that received the 5-day *vs*. the 7-day CO-Synch + CIDR program ovulated follicles of similar diameter that tended to produce greater peak estradiol concentrations (Bridges *et al*., 2014). A logical explanation for this difference in estradiol concentrations is the extended period of stimulation by high frequency LH pulses. However, an additional factor that we think may also contribute to enhanced systemic estradiol with a 5-day *vs*. 7-day program is that removal of progesterone restraint of LH secretion occurs earlier relative to follicular wave emergence. With a 5-day, as compared to a 7-day program, follicles resulting from the new wave initiated after the first GnRH injection would be approximately 3 to 4 days post-emergence, *vs*. 5 to 6 days from emergence, respectively, at PGF and CIDR removal. It has been reported that growing dominant follicles, 4 days after emergence, have increased intra-follicular estradiol concentrations and capacity to produce estradiol *in vitro* than non-atretic dominant follicles at a time later in the follicular wave (Valdez *et al*., 2005). Furthermore, it has been demonstrated that concentrations of estradiol in the caudal vena cava were greater (Rhodes *et al*., 1995) at approximately 3 days after emergence of the first wave dominant follicle as compared to later in the lifespan of this follicle. Hence, extending proestrus and removing progesterone at a time when steroidogenic capacity of dominant follicles is optimal may both contribute to greater peak concentrations and/or an extended period of elevated estradiol during proestrus. The concentrations of estradiol present during the preovulatory period in cattle is increasingly recognized as a key factor that influences fertility (Bridges *et al*., 2012, 2013; Atkins *et al*., 2013; Jinks *et al*., 2013; Geary *et al*., 2013). We have concluded that a key impact of increased length of proestrus is to escalate preovulatory concentrations of estradiol in response to a longer period of LH stimulation and have demonstrated greater estradiol concentrations during proestrus and an increased timed-AI pregnancy rate in the 5-day as compared to the 7-day CO-Synch + CIDR program.

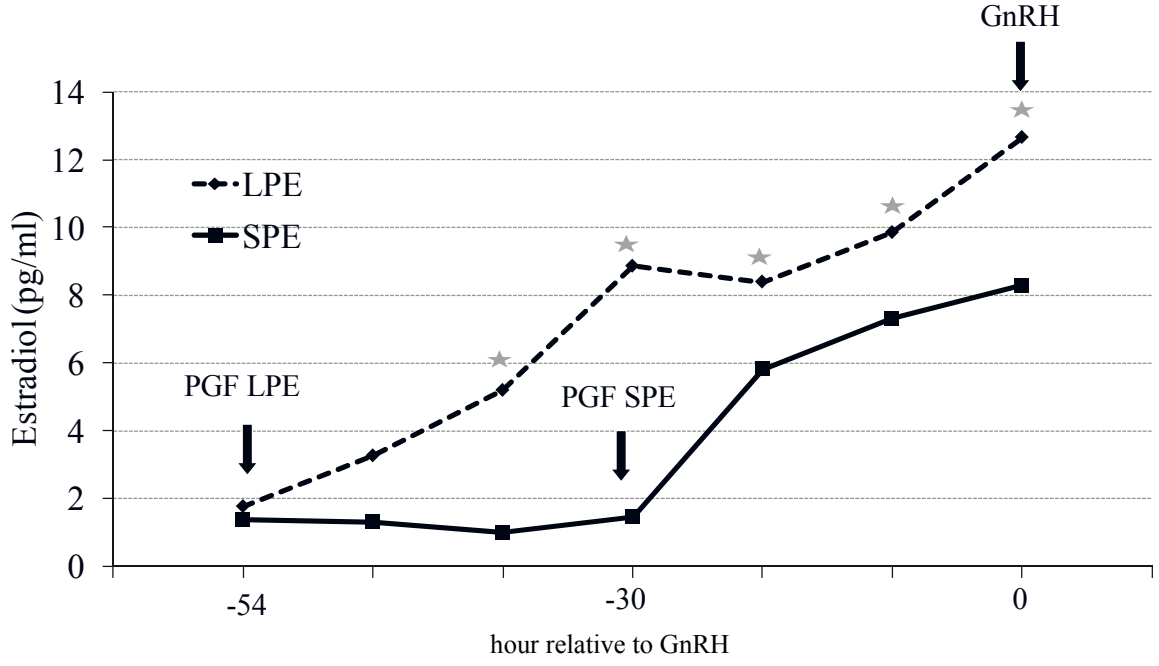

Figura 1. Concentrations of estradiol in cows that experienced either a long (LPE) or short (SPE) proestrus Adapted from Bridges *et al*., 2010.

Follicular growth and length of synchronization program

It has been demonstrated that the 7-day CO-Synch program results in a proportion of cows that ovulate follicles of smaller than typical diameter at timed-AI, which results in decreased fertility in these animals (Lamb *et al*., 2001; Perry *et al*., 2005). We and others have presumed that the smaller follicles at the time of synchronized ovulation are the result of spontaneous atresia of follicles and initiation of a new wave of follicular development during the latter stages of the interval between the initial GnRH treatment and PGF in females that do not respond to the first GnRH treatment. It has been reported that this is one of a variety of factors that contribute to the variation in diameter of follicles at the synchronized ovulation (Atkins *et al*., 2008, 2010). Reducing the length of CIDR treatment from 7 to 5 days would be expected to reduce the likelihood that this pattern of follicular growth would occur, and result in greater estradiol concentrations during proestrus in these females. Failure to ovulate to the first GnRH resulted in reduced preovulatory estradiol concentrations and progesterone concentrations during the subsequent luteal phase in the 7-day but not in the 5-d CO-Synch + CIDR program (Bridges *et al*., 2014). In a recent preliminary report (Dias *et al*., 2014), females which did not respond to the first GnRH administration in the 5-day CO-Synch + CIDR program had greater TAI pregnancy rates (65.0%; n = 163; P = 0.01) than those that did respond to GnRH-1 (51.5%). Further research regarding this finding, and whether a portion of the benefit of the 5-day program is the result of normal fertility in those females not responding to the initial GnRH, is necessary.

Age of follicles at induced ovulation

In the 5-day CO-Synch + CIDR program, the interval from follicle wave emergence at 1 to 2 days after the first GnRH, to induction of ovulation with the second GnRH (day 8) is 6 to 7 days. In the 7-d program, this interval is 8 to 9 days. We refer to this interval as follicle age, and due to the design of the 5-day program, younger follicles are induced to ovulate with the second GnRH as compared to the 7-day program. Variation in follicle age at ovulation does exist in spontaneously ovulating cows. For example, in spontaneously ovulating dairy cattle that have either 2 or 3 waves of follicular growth during their estrous cycle, the interval from follicle emergence to estrus or ovulation (age of the follicle) is greater by approximately 3 days in cows (Bleach *et al*., 2004) and 4 days in heifers (Sartori *et al*., 2004) with 2 follicular waves; and pregnancy rate to AI is lower when compared to cows with 3 follicular waves during the estrous cycle (Townson *et al*., 2002). Use of a 5 day interval between GnRH and PGF increased pregnancy rate in lactating dairy cows (Santos *et al*., 2010) and Cerri *et al*. (2009) demonstrated a greater proportion of good quality embryos collected from lactating dairy cows that were induced to ovulate younger follicles; within the range normally observed in spontaneously ovulating females. The cumulative interpretation of reports in lactating dairy cows suggests that age of the follicle is a significant source of variation in fertility. We have recently completed two experiments to directly address the effect of age of the ovulatory follicle on fertility in cattle and tested the hypothesis that conception rate to AI after ovulation of a younger follicle would be greater in beef heifers after spontaneous ovulation and in postpartum beef cows after either a spontaneous or GnRH-induced ovulation.

In the first experiment in heifers, luteal regression was induced with PGF either 2 (young follicle = YF) or 6 (mature follicle = MF) days after emergence of a new follicular wave and heifers were AI 12 h after expression of estrus (Abreu *et al*., 2014a). As expected, the interval from PGF to estrus was greater in the YF than MF group with some variation in this interval between locations (Table 2). Age of follicles at AI was greater by approximately 3 days in the MF group, and diameter of the ovulatory follicle was marginally greater in the MF than YF heifers. However, conception rate to estrus-AI did not differ between groups.

In postpartum cows (Abreu *et al*., 2014b), luteal regression was induced with PGF either 2.5 (young follicle = YF) or 6.5 (mature follicle = MF) days after emergence of a new follicular wave. Based upon the intervals to estrus in heifers (Abreu *et al*., 2014a), cows in the MF group were AI based upon estrus detection until 72 h after PGF with the cows not detected in estrus receiving GnRH and timed-AI at hour 72. In the YF group, estrus detection and AI was performed to hour 96, with timed-AI in the remaining cows at hour 96. Interval to estrus after PGF was approximately 24 h greater in the YF than MF treatment (Table 3). This resulted in a difference in follicle age at AI of approximately 3 days (MF > YF) yet diameter of the ovulatory follicle did not differ between treatments. Pregnancy rate during the synchronization period did not differ between cows in the MF and YF treatments (Table 3).

As previously described, in cattle that initiate a new follicle wave after the first GnRH, age of the ovulatory follicle for a 5-day program, by design, is approximately 2 days less than with a 7-day CO-Synch + CIDR program. Results of experiments by Abreu *et al*. (2014a, b) suggest that age of ovulatory follicles, in itself, for females that respond to the first GnRH may not be a substantial source of variation in timed-AI pregnancy rate.

Table 2. Effect of treatment (Trt) on estrous response, proestrus interval, follicle age and size at AI (Mean ± SE), and conception rate in both locations.

	Trt[1]	n	Estrous response (%)	Proestrus[2] interval (h)	Follicle age[3] at AI (day)	Follicle size at AI (mm)	Conception rate (%)
Montana	MF	53	92.5	55.8 ± 2.7[a]	8.3 ± 0.11[a]	11.0 ± 0.18[a]	63.3
	YF	75	90.7	67.4 ± 1.6[b]	4.8 ± 0.06[b]	10.4 ± 0.15[b]	64.7
Ohio	MF	77	87.0	53.7 ± 2.2[a]	8.2 ± 0.10[a]	-	64.2
	YF	75	90.7	78.5 ± 1.4[c]	5.3 ± 0.06[c]	-	69.1

[a-c]Values with different superscripts in the same column differ (P < 0.01). [1]MF = mature follicle; YF = young follicle. [2]Proestrus interval was defined as the interval from prostaglandin F2α (PGF2α) administration to estrus. [3]Follicle age was defined as the interval from estradiol benzoate (EB) administration to 12 h after estrus minus 3 days for new follicle wave formation to occur for heifers that received PGF2α either 5 (YF) or 9 days (MF) after EB. Adapted from Abreu *et al*. (2014a.

Table 3. Effect of treatments on response variables (mean ± SE) in beef cows.

Variable[1]	Mature follicle	Young follicle	P- value
Estrous response within 72 h (%)	76.3	47.7	< 0.01
Estrous response from PGF to TAI - 72 *vs*. 96 h (%)	76.3	88.6	< 0.01
Interval from PGF to estrus (h)	57.5 ± 1.6	78.9 ± 0.8	< 0.01
Follicle age at AI (day)	9.32 ± 0.04	6.26 ± 0.02	< 0.01
Follicle diameter at AI (mm)	13.1 ± 0.2	12.9 ± 0.1	> 0.10
Follicle growth rate - PGF to AI (mm/day)	0.95 ± 0.07	1.14 ± 0.04	< 0.05
Follicle growth rate, 5.5 d after EB to PGF (mm/day)	0.77 ± 0.06	n/a	-
Pregnancy rate (%)	72.0	67.1	> 0.10
Progesterone concentration (~ 7 d) after AI, ng/ml (G1 only)	3.56 ± 0.21	3.85 ± 0.13	> 0.10

[1]TAI = timed AI; EB = estradiol benzoate; G1 = group 1; PGF2α = prostaglandin F2α. Adapted from Abreu *et al*. (2014b).

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Is the number of antral follicles an interesting selection criterium for fertility in cattle?

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Abstract

Recent studies indicate that the antral follicle population may be of paramount importance to improve reproductive performance in cows. There is already an agreement that the antral follicle count (AFC; follicles ≥3 mm in diameter) is a highly variable trait among animals, but with high repeatability in the same individual. Thus, females can be classified into low, intermediate or high AFC. Several studies in *Bos taurus* show a positive correlation between AFC and fertility parameters, such as increased quantity and quality of embryos, better pregnancy rates, higher progesterone levels, among others. However, there is still no consensus on AFC in *Bos indicus* females and *indicus-taurus*. This article aims to discuss the main aspects related to the population of antral follicles and its relation to the reproductive performance associated with the most common techniques in assisted reproduction (timed artificial insemination, *in vitro* embryo production, embryo transfer and superovulation).

Keywords: antral follicle count, embryo production, follicular dynamics, pregnancy rate, ultrasonography.

Introduction

High genetic quality animals can be multiplied efficiently using reproductive biotechnologies such as artificial insemination and embryo production. These biotechnologies are useful strategies and known worldwide for improving genetics and productivity of flocks over a short period (Mapletoft and Hasler, 2005; Boni, 2012; Hansen, 2014).

Recently, there have been an increasing interest in studies concerning antral follicle count (AFC) and its influence on the reproductive performance in cattle, as well as its applications in reproductive biotechnologies (Ireland *et al.*, 2011; Pontes *et al.*, 2011; Rico *et al.*, 2012; Silva-Santos *et al.*, 2014a, b). Such fact may result in immense repercussions on the current scenario of animal reproduction, considering the significant increase in the world's embryo production. Despite several favorable results about AFC in *Bos taurus*, many aspects of reproductive physiology remain unknown. Considering AFC in *Bos indicus* there are many points to be addressed, particularly the impact on fertility when using *in vitro* embryo production (IVEP), timed artificial insemination (TAI) and timed embryo transfer (TET).

The high variability in the population of antral follicles is a hallmark in cattle (Burns *et al.*, 2005) with low, intermediate or high AFC (Santos *et al.*, 2012, 2013; Mendonça *et al.*, 2013). Despite the high variability among animals, there is a high repeatability of the number of follicles observed in the same individual through evaluations carried out during a period (Burns *et al.*, 2005; Ireland *et al.*, 2007, 2008, 2009).

This constancy in AFC in the same individual becomes a strategic resource for the possibility of classifying an animal by the AFC with a single ultrasound examination. For *taurus* animals, AFC is directly correlated with the size of the ovarian follicular reserve (Ireland *et al.*, 2011), which was not proven in indicus females, considering fetuses, heifers and cows (Silva-Santos *et al.*, 2011). However, other factors such as genetics (Walsh *et al.*, 2014), maternal environment, nutritional status and healthiness (Ireland *et al.*, 2011; Evans *et al.*, 2012) also appear to influence the AFC. For example, the nutritional status and the metabolic rate were mentioned as factors which affect the follicular growth, oocyte quality and secretion of reproductive hormones in cattle (Jimenez-Krassel *et al.*, 2009; Mossa *et al.*, 2010; Evans *et al.*, 2012).

The AFC may also influence the production of cattle embryos, both *in vivo* and *in vitro*, especially as the number of embryos produced by donor but also in process efficiency, with higher rates for high AFC animals (Ireland *et al.*, 2008; Santos *et al.*, 2014; Silva-Santos *et al.*, 2014a).

The importance of the AFC and its relationship with pregnancy rates must also be emphasized. Studies conducted with *taurus* females had higher pregnancy rates for high AFC females (Cushman *et al.*, 2009; Evans *et al.*, 2012; Mossa *et al.*, 2012). However, in recent studies with *indicus-taurus* and *indicus* animals, a better performance regarding pregnancy rates was not observed in high-AFC animals (Mendonça *et al.*, 2013; Santos *et al.*, 2014). Surprisingly, some data suggest a better performance regarding pregnancy rates for low AFC cows (Santos *et al.*, 2013).

In addition to ultrasound, the measurement of the concentration of anti-Mullerian Hormone (AMH)

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can also be used for selection of females according to the size of follicular population, since AMH has been considered as a factor highly correlated with the number of antral follicles and healthy oocytes (Hehenkamp *et al.*, 2006; Ireland *et al.*, 2011).

Considering the impact of reproductive biotechnology in genetic improvement and doubts as to the effect of antral follicle count on the reproductive performance in *indicus* animals, this review aims to discuss the main aspects of the AFC related to the number of antral follicles and its relationship with follicular dynamics, TAI, OPU/IVF and SOV.

Antral follicles count and its repeatability

The number of ovarian antral follicles is highly variable in different species (Burns *et al.*, 2005; Ireland *et al.*, 2007; Pontes *et al.*, 2011; Santos *et al.*, 2012), but in cattle, there is a high repeatability in the same individual (Burns *et al.*, 2005). Thus, the possibility of selecting *taurus* females by ultrasonography based on the AFC was demonstrated, due to the high degree of repeatability between individuals, regardless of race, age, breeding season, lactation or pregnancy conditions (Burns *et al.*, 2005). The same situation of AFC repeatability was demonstrated in *indicus* blooded animals; evaluating the same females for several months it was possible to identify the high correlation (0.90-0.92) in *taurus-indicus* weaned and yearling females (Santos *et al.*, 2012; Silva-Santos *et al.*, 2014a). Therefore, a single routine ultrasound examination can identify females with low, intermediate or high AFC, according to findings also observed in Nelore females (Morotti *et al*., 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data).

Antral follicles count and embryo production

Considering the importance of the production of embryos for genetic improvement of livestock, it is noted that there is a great variability in the number of embryos produced by the donor, in both *in vitro* and *in vivo* methods (Pontes *et al.*, 2009, 2010, 2011). The high variability in oocyte recovery rate and superovulatory response are important factors that affect the success of the bovine embryo production (Taneja *et al.*, 2000; Ireland *et al.*, 2007, 2011; Silva-Santos *et al.*, 2014a). In this context, several studies with *taurus* animals and some studies with *indicus*-blooded animals have shown that a greater number of follicles is associated with quantitative benefits to the success of IVEP and SOV (Taneja *et al.*, 2000; Singh *et al.*, 2004; Ireland *et al.*, 2008; Silva-Santos *et al.*, 2014a). For *indicus-taurus* animals, the average number of embryos per collection was also higher for females with high compared to low AFC animals (6.9 ± 5.3 *vs.* 1.9 ± 2.1; $P < 0.05$). Therefore, this study reinforces the evident concept that there are quantitative benefits of embryo production according to the number of antral follicles (Table 1).

Table 1. Reproductive performance of *Bos taurus-indicus* females with high (G-High, follicles ≥40) or low (L-Low, ≤10 follicles) antral follicles count comparing the production of embryos *in vitro* (ovum pick up/*in vitro* production - OPU/IVP) and *in vivo* (superovulation (SOV).

Varibles	G-High (n = 20)	G-Low (n = 20)
Antral follicles (n)	47 ± 6	9 ± 3
Total of oocytes retrieved	738[a]	116[b]
Percentage of viable oocytes (%)	58.94 (435/738)	55.17 (64/116)
Cleavage rate (%)	61.25 (452/738)	56.03 (65/116)
Blastocyst rate (%)	16.53 (122/738)	9.48 (11/116)
Total embryos / OPU/IVP (n)	6.10 ± 4.51[aA] (122/20)	0.55 ± 0.83[bB] (11/20)
Total structures recovered / collected (n)	8.80 ± 6.78[a] (176/20)	2.25 ± 2.63[b] (45/20)
Total embryos / collected (n)	6.95 ± 5.34[aA] (139/20)	1.9 ± 2.13[bA] (38/20)
Frozen proportion (%)	78.42[a] (109/139)	89.47[a] (34/38)

The values followed by different superscript letters ([a, b]) within the same line (G-G *vs*. high-low) or ([A, B]) within the same column (the OPU/IVP *vs*. SOV) were significantly different ($P \leq 0.05$). Adapted from Silva-Santos *et al*. (2014a).

Similar results were obtained with *taurus* donors by Ireland *et al*. (2007), with low AFC females presenting a lower mean number of *in vitro*-produced embryos compared with high AFC animals (1.3 *vs*. 4.9 embryos). In *in vivo* production of the embryos, low AFC donors (<15 follicles) produced fewer structures

when compared with high AFC females (>25 follicles), with means of 3.8 *vs*. 5.4 embryos, respectively.

Contradictory results for *indicus* animals were found by our research team. In an experiment with Nelore donors (n = 66), they were separated into groups of high AFC (AFC > 40 follicles); medium AFC (18 to 25 follicles) or low AFC (AFC < 7 follicles), and the oocytes were fertilized with sperm from a single bull. In addition to the expected quantitative superiority, a qualitative superiority was observed according to the AFC. Cows with high, medium and low AFC had blastocyst rates of 42, 32 and 13%, respectively (P < 0.05; Santos *et al.*, 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data). However, in a recently concluded study, this pattern was not confirmed. From a larger sample of Nelore cows (n = 356), oocytes were obtained from females of high (>92 follicles), medium (46-76 follicles) or low AFC (<31 follicles). All the oocytes were also fertilized with sperm from a single bull. There was no difference in cleavage and blastocyst rates: 40, 36 and 38% for high, medium and low AFC, respectively (Rosa *et al.*, 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data).

In addition to the results of embryo production and pregnancy rates, mRNA expression of genes associated with follicular and oocyte viability seem to show similar patterns in zebu females of high, medium or low AFC. There was no significant difference in the expression of genes related to steroidogenesis (CYP19 and STAR), cell proliferation and differentiation (TGFB1, LIFRa and BMPR2) and hormonal production or response to hormones (AMH, FSHr, PGR and PGRMC) in cumulus and granulosa cells of Nelore females (Rosa *et al.*, 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data). The contradiction of such results in the same research team reinforces the complexity of this issue.

It is noted that AFC is not the only aspect to quantitatively interfere in embryo production, considering that donors respond to OPU/IVF or SOV according to variables not yet fully understood. In this context, Pontes *et al*. (2009) reported that some donors provided better results on *in vitro* or *in vivo* techniques, regardless of the number of follicles (Table 2).

Table 2. Variations in embryo production rates of six Nelore (*Bos indicus*) donors obtained by *in vitro* procedures (ovum pick up/*in vitro* production - OPU/IVF) or *in vivo* (superovulation and embryo collection - SOV).

Variables	Donor					
	I	II	III	IV	V	VI
Total procedures OPU/IVF	5	5	4	4	5	5
Mean oocytes per OPU	36.6	25.6	49	29.7	22.8	16
Mean viable oocytes per OPU	32.2	23.4	45.2	26	19.6	14.4
Mean embryos per OPU	15.6	10.4	24.1	10.3	6.8	3.8
Mean pregnancy per OPU	4.8	2.8	9.25	4.3	2.2	1
Total procedures SOV/collection	2	3	2	2	2	3
Mean embryos per SOV	10	4.3	6.5	2	12.5	5.3
Mean pregnancy per SOV	5.5	2	1	1.5	6.5	1.3

Adapted from Pontes *et al*. (2009).

Despite these individual variations according to the technique, there is a consensus that the quantitative advantages of the high AFC donor should be exploited. Thus, the variation in the number of follicles per donor is currently a very important aspect for the commercial programs of embryos production. A screening method for selection of donors through an ultrasonographic pre-evaluation is commonly used. The selected donors are generally those with high AFC or a high number of oocytes, which are directly correlated variables. The impact on the final number of pregnancies varies widely, as shown in Table 3.

Table 3. Production of embryos and pregnancies according to the number of oocytes obtained by OPU/IVP (n = 656) from Nelore donors (n = 317). The values (mean ± SD) are presented per donor.

Donors according to oocyte production	N°. viable oocytes	N°. viable embryos	N°. pregnancy 30 days	N°. pregnancy 90 days
Elevated (n = 78)	47.06 ± 1.6^{a}	15.06 ± 0.86^{a}	5.62 ± 0.54^{a}	5.52 ± 0.81^{a}
High (n = 80)	24.95 ± 0.33^{b}	9.17 ± 0.63^{b}	3.63 ± 0.36^{b}	3.32 ± 0.33^{b}
Medium (n= 79)	15.57 ± 0.26^{c}	6.00 ± 0.39^{c}	2.10 ± 0.21^{c}	1.92 ± 0.20^{b}
Low (n = 80)	6.31 ± 0.38^{d}	2.42 ± 0.25^{d}	0.92 ± 0.13^{d}	0.85 ± 0.13^{c}

$^{a-d}$Within a column, the mean values with no common superscript differ significantly ($P \leq 0.05$). Adapted from Pontes *et al*. (2011).

Despite the unquestionable quantitative advantages in number of embryos for the donor selection method for OPU/IVF based in the AFC, the impact of this criterion on other aspects of fertility and production of meat or milk is not well known for *indicus* animals. Thus, until more studies are performed, the choice of donor based on the number of follicles should be performed only after assessing the genetic merit for production traits of the donor.

Number of antral follicles and anti-Mullerian hormone

AMH is a hormone that belongs to the family of growth factor -β, and is produced by granulosa cells from healthy growing follicles. Its expression is elevated in granulosa cells of small antral follicles and decreases during the follicular growth. The high concentrations of AMH are positively associated with follicular size of mice, women and cows' ovaries population. In the latter, the concentration of AMH is highly correlated with the number of antral follicles and healthy oocytes, thus can be considered a viable endocrine marker of AFC in cattle (Batista *et al.*, 2014).

Bovine females with high AFC (>25 follicles) have higher circulating AMH concentrations in comparison to the females with low AFC (<15 follicles; $P < 0.01$), with a high correlation between the average concentration of AMH and the mean AFC to *Bos taurus* cattle ($r = 0.88$, $P < 0.001$; Ireland *et al.*, 2008). When comparing *taurus* and *indicus* females, both beef and dairy cattle, AMH concentration was also positively correlated with AFC in *indicus* (Nelore, ranging from 0.56 to 0.68) and *taurus* (Holstein, ranging from 0.73-0.90). Regardless of the genetic group, females with high AFC showed higher AMH concentrations (0.57 ng/ml in *taurus vs*. 1.20 ng/ml in *indicus*) than females with low AFC (0.06 and 0.78 ng/ml, respectively). Furthermore, there is evidence that AFC in *indicus* (low and high, 28 and 48 follicles, respectively) is higher than in *taurus* (low and high, 13 and 34 follicles, respectively; Batista *et al.*, 2014).

Currently, AMH is recognized as a reliable indicator of ovarian response to superovulation. Therefore, the determination of AMH concentration in donor cows may help predict the follicular and ovulatory responses to gonadotropic treatment (Rico *et al.*, 2009).

The AMH concentrations in blood or plasma may be determined by an ELISA test. In a study with Holstein cows over a year, the concentrations of AMH were constant and strongly correlated with the AFC. Donors with AMH concentrations below 87 pg/ml showed less than 15 large follicles by estrous cycle and low efficiency in the production of embryos. Thus, the determination of the concentration of AMH in bovine plasma could routinely be considered in procedures of OPU/IVF and SOV for identification of animals with the best embryo production potential (Rico *et al.*, 2012).

Number of antral follicles and progesterone

The production of progesterone has been linked to physiological activity of the corpus luteum and to ovary and uterus functionality, with direct impact on embryonic development and pregnancy in cattle (Pohler *et al.*, 2012). Low progesterone concentrations are associated with high rates of embryonic mortality, less healthy oocytes and slower growth of the endometrium in these females (Diskin and Morris, 2008). Low AFC *taurus* females showed low concentrations of progesterone during their oestrus cycle, in comparison with high AFC females. The lower circulating concentrations of progesterone in cows with low AFC were mainly attributed to decreased function of the corpus luteum, possible changes in the responsiveness of luteal cells to LH, a potential reduction STAR protein in the corpus luteum, diminished responsiveness of granulosa and luteal cells to 25-hydroxycholesterol and the reduced ability of granulosa cells of dominant follicles to undergo luteinization in order to produce progesterone (Jimenez-Krassel *et al.*, 2009). It is not clear why an ovulated follicle that turned into a CL would produce less P4 when it is in a low AFC animal. Mainly considering recent studies in *Bos indicus* in which the situation seems to be the opposite (Seneda et *al*., 2015; DCV-CCA-, Londrina, PR, Brazil; unpublished data). Considering this and other aspects, we believe in distinct pathways in *Bos taurus* and *Bos indicus* at least for some reproductive aspects. It has also been demonstrated that high AFC was positively associated with endometrial thickness. An increased endometrial thickness was associated with higher embryonic implantation rates (Basir *et al.*, 2002).

Antral follicle count and fertility parameters in beef heifers and cows

The low AFC in dairy *taurus* females (Holstein) was associated with several characteristics related to unfertility, such as smaller ovaries (Ireland *et al.*, 2008), less chance of pregnancy at the end of the breeding season (Mossa *et al.*, 2012), reduced responsiveness to the SOV treatment, fewer viable embryos (Singh *et al.*, 2004; Ireland *et al.*, 2007), lower circulating concentrations of progesterone and AMH (Ireland *et al.*, 2011; Evans *et al.*, 2012) and reduced endometrial thickness (Jimenez-Krassel *et al.*, 2009). In this context, animals with high AFC were proved superior in all of the mentioned aspects. Thus, it is assumed and substantiated that in Holstein cows a linear correlation exists between high AFC and indicators for reproductive efficiency.

This context, however, has not been proven in Zebu animals. Recent studies have shown that reproductive traits of *indicus* (Nelore) and *taurus-indicus* females (Braford) did not show improved performance associated with high AFC (Santos *et al.*, 2012, 2013; Mendonça *et al.*, 2013; Morotti *et al.*, 2014;

Santos *et al.*, 2015, DCV-CCA-UEL, Londrina, PR, Brazil, unpublished data). A higher rate of follicular growth and larger follicular diameters have been described in *indicus-taurus* (Santos *et al*, 2012) and *indicus* (Morotti *et al*, 2014) females with low-AFC. Also, dominant follicles with larger diameters have been described in Nelore females with low AFC (Morotti *et al*., 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data).

Considering the AFC bimonthly basis in 137 *taurus-indicus* females, there was no difference in the average body weight from nine to 24 months, or in the diameter of the uterine horn (Santos *et al.*, 2012). In a subsequent experiment, 71 females from same herd were subjected to a TAI protocol to determine the values of certain variables associated with follicular dynamics. The data are presented in Table 4.

Table 4. Mean ± SD values of follicular dynamics of *taurus-indicus* females (Braford) with different antral follicles counts (AFC; high, ≥40 follicles; medium, 17-23 follicles; and low, ≤10 follicles) after a protocol of ovulation synchronization.

Variables	High ≥40 follicles n = 24	Medium 17-23 follicles n = 24	Low ≤10 follicles n = 23
Antral follicle count on D5 (n)	47 ± 9.9[a]	24 ± 9.9[b]	9 ± 3.9[c]
Diameter of the largest follicle on D5 (cm)	0.66 ± 0.3	0.70 ± 0.2	0.80 ± 0.2
Ovulatory follicle diameter (cm)	1.15 ± 0.2[a]	1.27 ± 0.2[b]	1.32 ± 0.2[b]
Interval between device removal and ovulation (h)	69.33 ± 5.1	71.25 ± 3.0	70.50 ± 4.1
Ovulation rate (%)	75 (18/24)	67 (16/24)	70 (16/23)
CL diameter (cm)	1.93 ± 0.3	1.97 ± 0.3	2.04 ± 0.3

[a–c]Within a column, the mean values with no common superscript differ significantly (P ≤ 0.05). Adapted from Santos *et al*. (2012).

Unlike the results reported for Holstein cows (Ireland *et al.*, 2011; Evans *et al.*, 2012), the data in Table 4 show that Braford females of low and medium AFC had ovulatory follicles with greater diameters.

Regarding pregnancy rates of *indicus* (Nelore) females, two studies have shown pregnancy data after TAI, one with postpartum cows (n = 691) and the other with heifers at 24 months of age (n = 208). On day 8 of the TAI protocol, the ovaries were evaluated by ultrasonography and females were classified into groups of high, medium or low AFC. Pregnancy rates were evaluated by transrectal ultrasonography 30 days after TAI. The data, summarized in Table 5, demonstrate that low AFC cows were superior to medium AFC cows, while high AFC cows were similar to both. There were no differences for heifers. In short, it was not possible to establish the same relationship described for *taurus* females.

Table 5. Reproductive performance of Nelore (*Bos indicus*) females with high, medium and low antral follicle count (AFC) after a timed artificial insemination protocol.

Cows (n)	High ≥25 follicles (149)	Medium 11-24 follicles (400)	Low ≤10 follicles (142)	Total (691)
Antral follicles (n)	30.70 ± 5.66[a]	17.03 ± 3.28[b]	7.83 ± 2.42[c]	17.93 ± 8.45[b]
Pregnancy rate (%)	51.67[ab]	48.00[b]	60.50[a]	51.49[ab]
Heifers (n)	**High ≥30 follicles (38)**	**Medium 13-29 follicles (143)**	**Low ≤12 follicles (27)**	**Total (208)**
Antral follicles (n)	37.73 ± 7.05[a]	19.23 ± 4.29[b]	10.55 ± 2.17[c]	21.48 ± 9.47[b]
Pregnancy rate (%)	44.73	43.35	51.85	44.71

[a-c]Within a column, the mean values with no common superscript differ significantly (P ≤ 0.05). Data are presented as mean ± SD. Adapted from Santos *et al*. (2013) and Mendonça *et al*. (2013).

The association between AFC and pregnancy rates was also analyzed in *indicus* recipients after the transfer of *in vitro*-produced embryos. Cyclical Nelore heifers (n = 281, ECC 3.0 ± 0.5) underwent a classic protocol for ovulation synchronization before TET. The heifers received the embryos 17 days after the beginning of the treatment. There was no difference in pregnancy rate according to AFC (Table 6).

Table 6. Antral follicle count and pregnancy rates of Nelore (*Bos indicus*) recipients after TET of *in vitro* embryo production.

Heifers (n)	High ≥25 follicles (38)	Medium 11 follicles (136)	Low ≤3 follicles (75)
Antral follicles (n)	25.8 ± 7,4[a]	11.3 ± 2.9[b]	3.8 ± 1.3[c]
Pregnancy rate (%)	30.0	33.8	34.6

[a-c]Within a column, the mean values with no common superscript differ significantly (P ≤ 0.05).

Antral follicle count, carcass phenotype and genetic characteristics of heritability

In a recent study, heritability and the impact of environmental effects during pregnancy on AFC was evaluated in cattle. In the Holstein breed, this parameter had heritability of 0.31 ± 0.14 and 0.25 ± 0.13 for cows and heifers, respectively. The AFC was negatively associated with genetic merit for milk fat. The authors concluded that the AFC in Holstein females is an inherited genetic trait moderately affected by age and lactation status, but not the mother's milk production level during pregnancy (Walsh *et al*., 2014).

To *indicus-taurus* heifers (Braford, n = 270), the AFC was considered in a statistical model associated with phenotypic characteristics for selecting matrices. The variables considered were mean number of antral follicles, effect of contemporary group, age, birth weight gain at weaning, conformation at weaning, finishing at weaning precocity, musculature at weaning, weight gain from weaning to yearling, forming at yearling, precocity at yearling and musculature at yearling. For all parameters studied, the correlation values were very low (0.056 to 0.082; P > 0.05), pointing out that, for *taurus-indicus* animals, the antral follicle population has no association with the main selection criteria of matrices for beef herds (Morotti *et al*., 2015; DCV-CCA-UEL, Londrina, PR, Brazil; unpublished data).

Final comments

After all comments above, our general conclusion is: so far, it is not possible to apply the *Bos taurus* AFC model for *Bos indicus* females. While the AFC seems to be a very clear criterium for reproductive selection in *taurus*, we have found contradictory results in *indicus* cattle. There are results signaling for a better reproductive efficiency of low AFC animals, others to the medium AFC females and even, in the same model of *taurus* cows, data suggesting that there is a greater reproductive efficiency in high AFC zebu females.

One of the main challenges in the analysis of articles refers to the large variation of the classification criteria of high, medium or low follicle count. There are values considered as low AFC in one article that are considered as high AFC in other study, using the same category and the same breed. This situation makes it very hard to compare data.

Nevertheless, the inconstancy of the results shows the need to always prioritize genetic merit in the choice of a donor, and not AFC. Following this criterion is particularly important for oocyte donors. In *in vitro* embryo production, the quantitative advantage of number of structures tends to create a biased selection of donors with high AFC, because they make the entire IVEP chain more profitable. Considering the estimate that Brazil, as the world leader of this biotechnology, produces annually around 300,000 embryos *in vitro* (Viana *et al*., 2012) it is necessary to consider that an immediate benefit in the number of embryos should not supercede a strict criterion of genetic merit, which is a more important aspect in short, medium and long term in the selection of animals.

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Relationship between follicle population, AMH concentration and fertility in cattle

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Abstract

The size of ovarian follicular population evaluated by direct antral follicular count or endocrine markers can help determine the success of reproductive biotechnologies in cattle. However, although highly repeatable within animal, the antral follicular population (AFP) appears to be greatly variable across individuals. Therefore, laboratory methods that reliably predict AFP could have a significant value to select donor-cows for use in reproductive biotechnology and for genomic selection of animals with greater reproductive potential. Accordingly, the circulating levels of anti-Müllerian hormone (AMH) have been found to be associated with AFP and, thereby, identified as an important endocrine marker of superovulation response and *in vitro* embryo production in cattle. Moreover, a number of recent publications and ongoing studies are trying to determine whether circulating levels of AMH are correlated with fertility. This review summarizes recent information concerning AFP and its association with AMH, and the possibility of utilizing AMH as a marker for reproductive technologies and ultimately to enhance cattle fertility.

Keywords: anti-Müllerian, antral follicle, artificial insemination, embryo transfer, ovarian response.

Introduction

Genetic selection and reproductive efficiency are key factors for the success of the dairy and beef industries. Reproductive technologies, such as ovum-pick-up (OPU) and *in vitro* embryo production (IVEP) can rapidly enhance genetics of dairy and beef cattle through both the female and male linage. However, in females, the success of these techniques is highly dependent upon individual physiological characteristics of the animal such as ovarian antral follicle population (AFP). Therefore, the efficiency of IVEP can be compromised by the large variability of AFP among donors, despite the high repeatability within animal (Burns, 2005; Ireland *et al*., 2007).

Ovarian antral follicle numbers are positively associated with a variety of indirect measures of fertility in cattle such as ovarian function (Ireland *et al*., 2008, 2009; Jimenez-Krassel *et al*., 2009), superovulation responses (Kawamata, 1994; Cushman *et al*., 1999; Singh *et al*., 2004), *in vitro* blastocyst production (Taneja *et al*., 2000; Pontes *et al*., 2009), fertility (Erickson *et al*., 1976; Maurer and Echternkamp, 1985; Oliveira *et al*., 2002; Mossa *et al*., 2012) and herd longevity (Jimenez-Krassel *et al*., 2015). Interestingly, AFP has also been associated to several blood compounds, including circulating concentrations of insulin, insulin-like growth factor I (IGF-1), and anti-Müllerian hormone (AMH; Alvarez *et al*., 2000; Fortune *et al*., 2010; Satrapa *et al*., 2013; Batista *et al*., 2014; Sales *et al*., 2015)

In cattle, circulating AMH concentration can help field veterinarians to predict AFP in ovaries (Ireland *et al*., 2008; Rico *et al*., 2009; Batista *et al*., 2014), response to superovulation treatments (Rico *et al*., 2009; Monniaux *et al*., 2010; Souza *et al*., 2015), and more recently as a marker to predict IVEP performance of *Bos taurus* (Gamarra *et al*., 2014; Guerreiro *et al*., 2014; Vernunft *et al*., 2015) and *Bos indicus* breeds (Guerreiro *et al*., 2014). In addition, studies performed in the last decade have also indicated that cows with lower number of antral follicle counts have lower fertility (Mossa *et al*., 2012). Therefore, because circulating AMH is an indirect measure of ovarian reserve, represented by the size of the ovarian follicle pool, later studies have explored the use of AMH to predict field fertility in cattle (Ribeiro *et al*., 2014; Jimenez-Krassel *et al*., 2015). However, the value of AMH on predicting field fertility may vary according to the type of reproductive management employed in the farm, since it appears that AMH was not associated to field fertility in cows bred following the use of timed AI protocols (Ribeiro *et al*., 2014). Hence, the present review aims to discuss some key points related to AMH and antral follicle population, superovulation responses, OPU-IVEP and field fertility.

Anti-Müllerian hormone (AMH) and antral follicular population (AFP)

AMH is a member of the TGFβ superfamily of growth factors (Cate *et al*., 1986) first described to have an important role in sex differentiation in early fetal life (Lee *et al*., 1996; Rajpert-De Meyts *et al*., 1999). In females, AMH is produced by granulosa cells mainly from pre-antral and early antral follicles (Durlinger *et al*., 2002). Therefore, despite the fact that the number of ovarian follicles is variable across females and yet highly repeatable within individuals, AMH is a reliable endocrine marker of ovarian reserve (entire population of follicles in ovaries; (Ireland *et al*., 2007, 2008;

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Monniaux *et al*., 2012). In this context, AMH have been correlated with AFP in cattle of different genetic groups (Fig. 1 and 2; Baldrighi *et al*., 2013; Batista *et al*., 2014) and categories (Fig. 3; Batista *et al*., 2015; FMVZ/USP, São Paulo, SP, Brazil; unpublished data). Moreover, AMH concentration appears to vary a lot across cattle breeds following the pattern in terms of AFP (Batista *et al*., 2014; Ribeiro *et al*., 2014) and there seem to be a different AMH-threshold for the differing genetic groups when trying to classify animals into differing classes of AFP (Batista *et al*., 2014; Guerreiro *et al*., 2014). For example, in a recent study *Bos indicus* heifers showed greater AFP and AMH concentration compared to *Bos taurus* heifers (Batista *et al*., 2014).

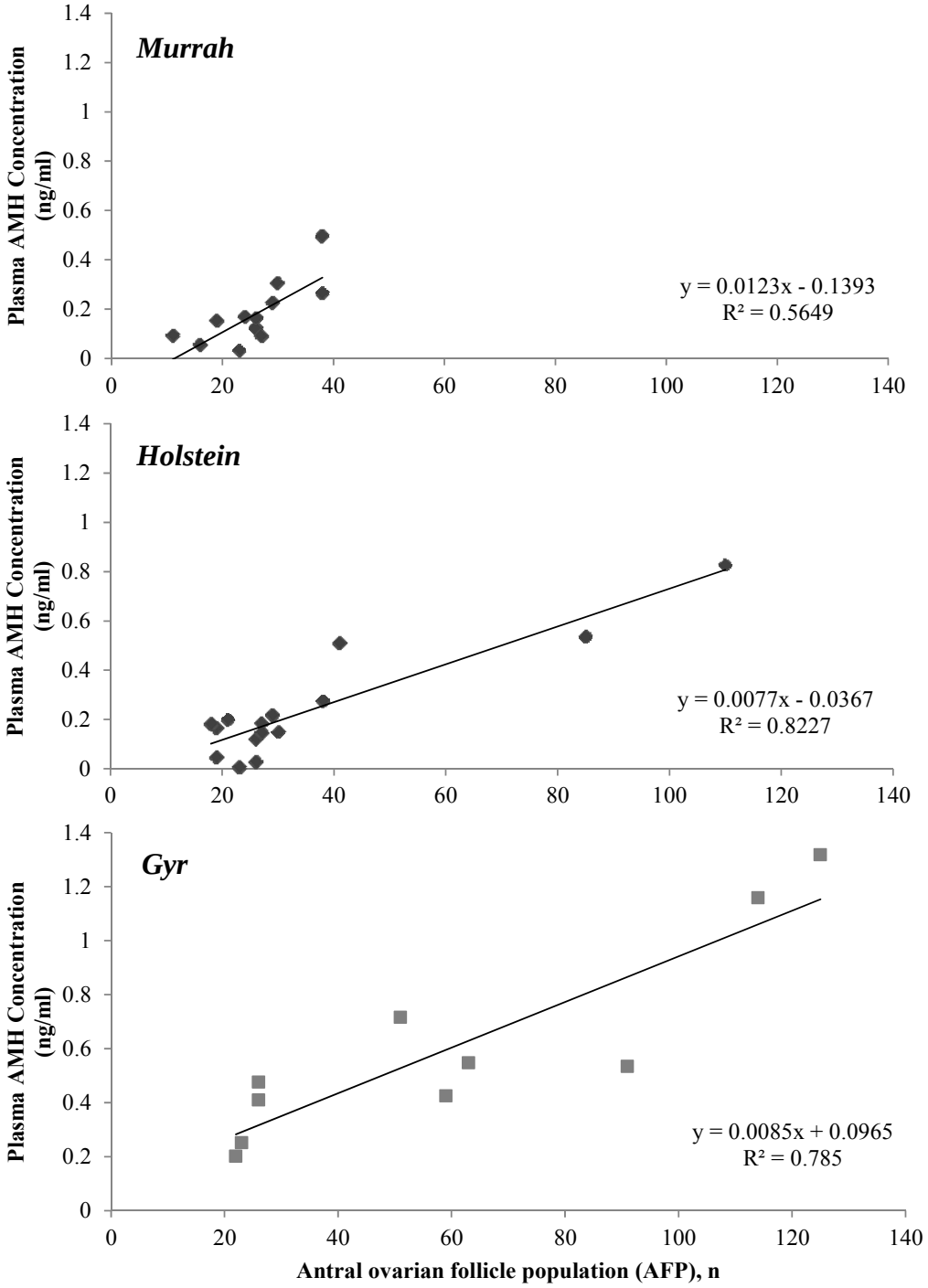

Figure 1. Relationship between the ovarian antral follicle population (AFP) counted at day of ovulation and the plasma anti-Mullerian hormone (AMH) concentration in Murrah (*Bubalus bubalis*, n = 13), Holstein (*Bos taurus*, n = 15) and Gyr (*Bos indicus*, n = 10 heifers). Adapted from Baldrighi *et al*. (2013).

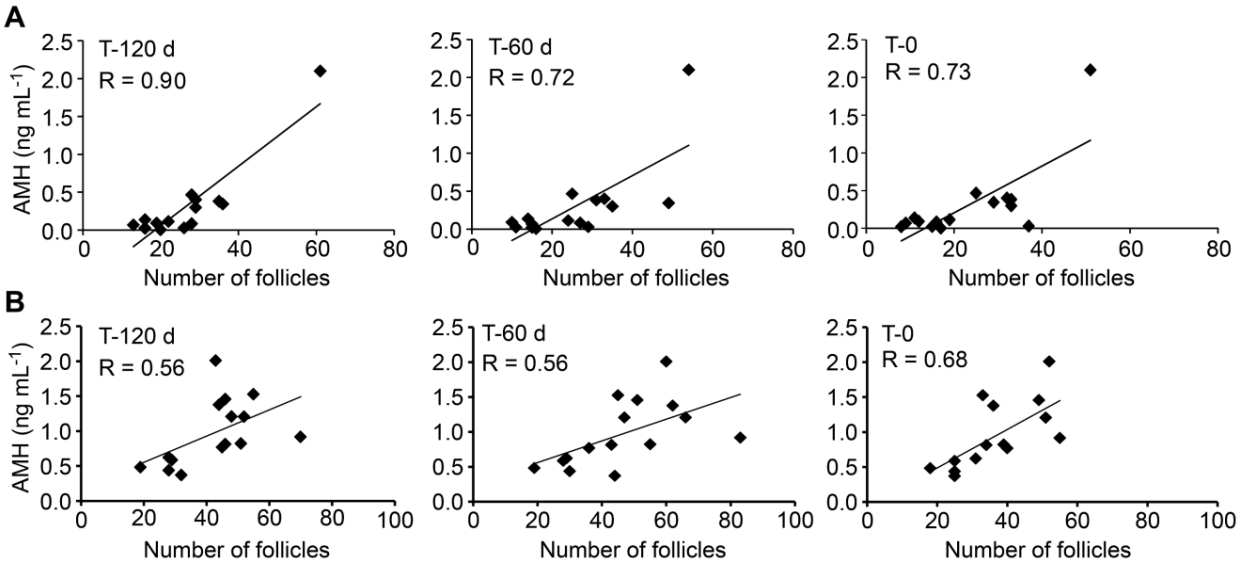

Figure 2. Relationship between the numbers of follicles counted at T-120, T-60, and T0 and plasma AMH concentration (ng/ml; T0) in Holstein heifers (n = 16; A) and Nelore heifers (n = 16; B). Ovarian antral follicular population (AFP) was evaluated three times at 60-day (d) intervals (T-120, 120 days before plasma AMH determination; T-60, 60 days before; and T0, at the time of plasma AMH determination). Blood samples were collected by jugular venipuncture on day T0 of the experimental design. Adapted from Batista *et al*. (2014).

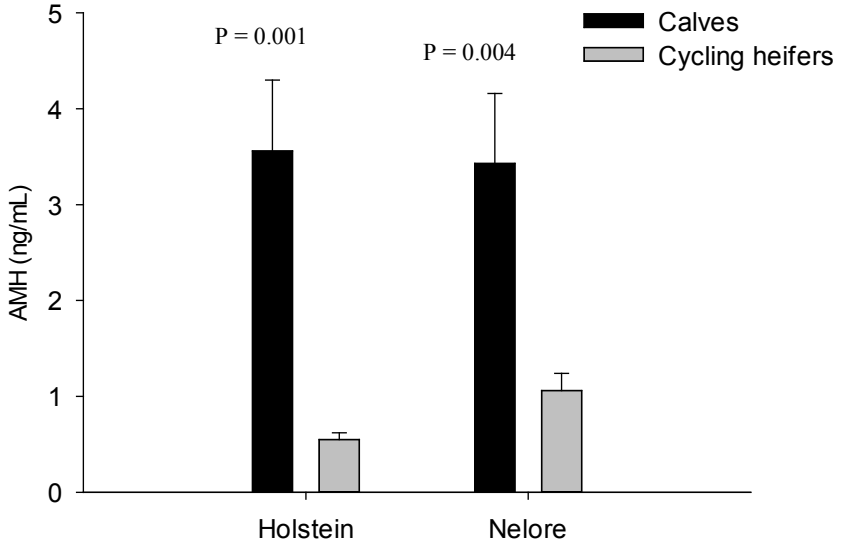

Figure 3. AMH plasma concentration (ng/ml) in calves (aging 2 to 4 months, Holstein: n = 24 and Nelore: n = 30) and cycling heifers (Holstein: n = 10 and Nelore: n = 12). Adapted from Batista *et al*. (2015); FMVZ/USP, São Paulo, SP, Brazil; unpublished data.

A practical important advantage in utilizing AMH instead of direct follicle count with an ultrasound to predict AFP is that AMH levels will vary minimally during the estrous cycle (Rico *et al*., 2009; Ireland *et al*., 2010; Souza *et al*., 2015), therefore blood samples can be taken at any time to evaluate circulating AMH. One exception would be the period just after superstimulatory treatments with FSH, in which the plasma AMH concentration appears to be greater than normal physiological levels (Rico *et al*., 2009, 2012). It is believed that this increase in AMH concentration following FSH treatment may be due to growth of small follicles that were not detected by ultrasonography (Rico *et al*., 2009, 2012). Alternatively, we can't rule out the possibility that FSH treatment may have increased AMH secretion by granulosa cells. However, this hypothesis needs further investigation (Rico *et al*., 2009, 2012).

Additionally, although AMH is produced mostly by early antral follicles, another intriguing biological aspect of AMH in the reproductive cycle in females is its involvement in mechanisms that inhibit activation of primordial follicles from entering the wave emergence pool (Durlinger *et al*., 2002; Fortune *et al*., 2010). Despite the larger AFP and AMH concentration observed in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) cattle (Batista *et al*., 2014), a previous report demonstrated that the primordial follicular populations were lower in *Bos indicus* than *Bos taurus* heifers (Silva-Santos *et al*., 2011). Additionally, ovaries of AMH null mice contained significantly more early atretic follicles (Visser *et al*., 2007). Therefore, it may be that the high AMH plasma concentration in *Bos indicus* heifers contributes to lower rates of follicular atresia.

Based on the finding that AMH can inhibit activation of primordial follicles from entering the wave emergence pool, it seems to avoid the premature depletion of the follicular population in the ovary. AMH concentrations decrease in parallel to the number of ovarian follicles as rodents (Kevenaar *et al*., 2006) and women (Piltonen *et al*., 2005) age. In agreement with that, in a recent study from our research group we have found greater plasma AMH concentrations in calves compared to cycling heifers in *Bos indicus* and *Bos taurus* cattle (Batista *et al*., 2015, FMVZ/USP, São Paulo, SP, Brazil, unpublished data).

Nutritional factors such as vitamin-D status in humans (Dennis *et al*., 2012) as well as negative energy balance in cattle (Souza *et al*., 2014) seem to have some influence in circulating AMH. Recently propylene glycol drenches administrated in Holstein heifers increased the number of follicles and blastocyst quality in heifers that had greater AMH concentration, but it had no impact in heifers with lower AMH concentration (Gamarra *et al*., 2014). Obviously, more research is urgently needed to try to manipulate circulating AMH through different feeding strategies.

AMH and superovulation/IVF response

Previous studies observed a strong positive relationship between circulating AMH and *in vivo* embryo production following superovulation in dairy cattle (Monniaux *et al*., 2010; Rico *et al*., 2012; Souza *et al*., 2015). AMH has been correlated with number of large follicles after superstimulation, number of CL after superovulation and number of embryos produced (Monniaux *et al*., 2010; Souza *et al*., 2015) in primiparous and multiparous cows (Souza *et al*., 2015) - shown in Fig. 4. Additionally, the type of blood anti-coagulant factor may influence AMH measurements and it appears to be an important detail when trying to interpret AMH results. For instance, a threshold of 87 pg/ml (samples collected with heparin) and 123 pg/ml (samples with EDTA) have been proposed to identify dairy cows producing less than 15 ovulatory follicles after FSH treatment and near the time of estrus. Thus, measuring AMH before enrolling cows in FSH programs will likely allow practitioners to improve numbers of embryos produced and, thereby, reduce costs per embryo produced.

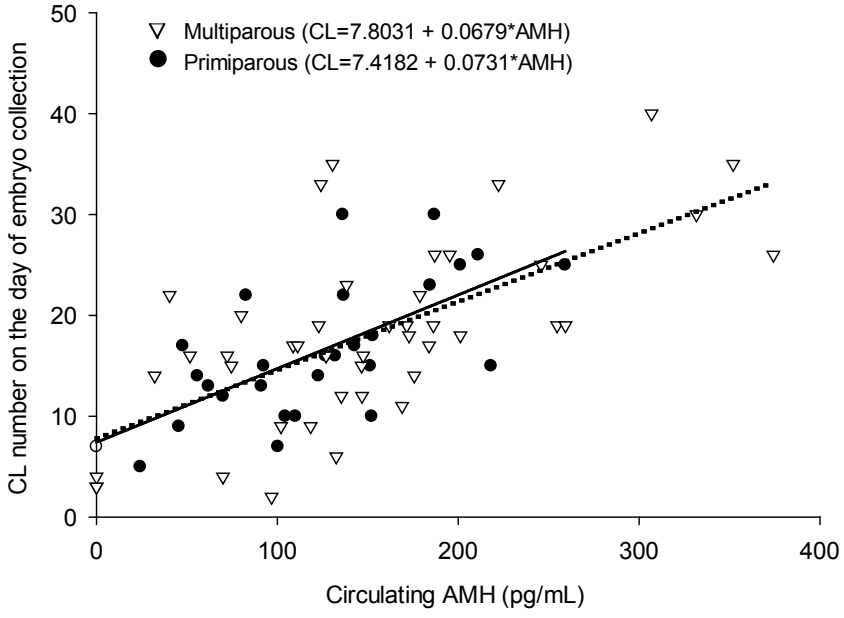

Figure 4. Average circulating AMH (pg/ml) and number of CL structures on the day of embryo collection for primiparous and multiparous dairy cows. Adapted from Souza *et al*. (2015).

In vitro embryo production has astonishingly increased in the last decade. Currently, 40.6% of the total embryo production in the world (1,275,874 embryos) are represented by *in vitro* embryo production (International Embryo Transfer Society - IETS, 2014). Therefore, ovum pick-up (OPU) and *in vitro* embryo production (IVEP) are important tools that can be used to drive genetic progress. However, success of IVF technology can be limited in animals that have lower numbers of follicles in the ovary. In that regard, recent studies have identified that AMH can be an interesting endocrine marker to select donors with the greatest potential to serve as donors for *in vitro* embryo production (Gamarra *et al*., 2014; Guerreiro *et al*., 2014; Vernunft *et al*., 2015). In agreement with these findings, plasma AMH in *Bos indicus* and *Bos taurus* heifers have been found to have a positive correlation with total follicles aspirated, total cumulus oocytes complexes (COCs) retrieved, number of COCs cultured, and number of embryos produced per OPU session (Fig. 5 and 6, and Table 1). However, some variables related to *in vitro* embryo development (cleavage and blastocyst rates) do not seem to have any correlation with circulating AMH (Fig. 5, and 6 and Table 1; Guerreiro *et al*., 2014).

Because genomic information allows producers to know genetic merit of their animals at early ages, we have recently explored the possibility of producing embryos retrieved from young female calves that were only 2-4 months old. Our preliminary results indicate that AMH concentration is once again a very useful marker to predict IVP performance of *Bos taurus* and *Bos indicus* calves (Batista *et al*., 2015; FMVZ/USP, São Paulo, SP, Brazil; unpublished data). In most situations, because examining ovaries from very young calves with an ultrasound can be difficult and unpractical, the determination of circulating AMH concentration in this animal category can be an important tool to select best oocyte-donors for *in vitro* embryo production, overcoming some of the technical limitations involved in utilizing an ultrasound in young calves. Therefore, we forecast that with the availability of genomic technology for the identification of animals with superior genetics at early ages and AMH measurement to facilitate identification of best oocyte-donors, that the use of calves as oocyte donors has its place in IVP programs and will allow faster genetic gains by dramatically decreasing generation intervals (Armstrong *et al*., 1992; Lohuis, 1995; Camargo *et al*., 2005).

Altogether, an increasing body of evidence to date have shown that circulating AMH is highly correlated with AFP, and it appears to be an interesting endocrine marker to identify best donors for IVEP, regardless of the genotype background and animal age.

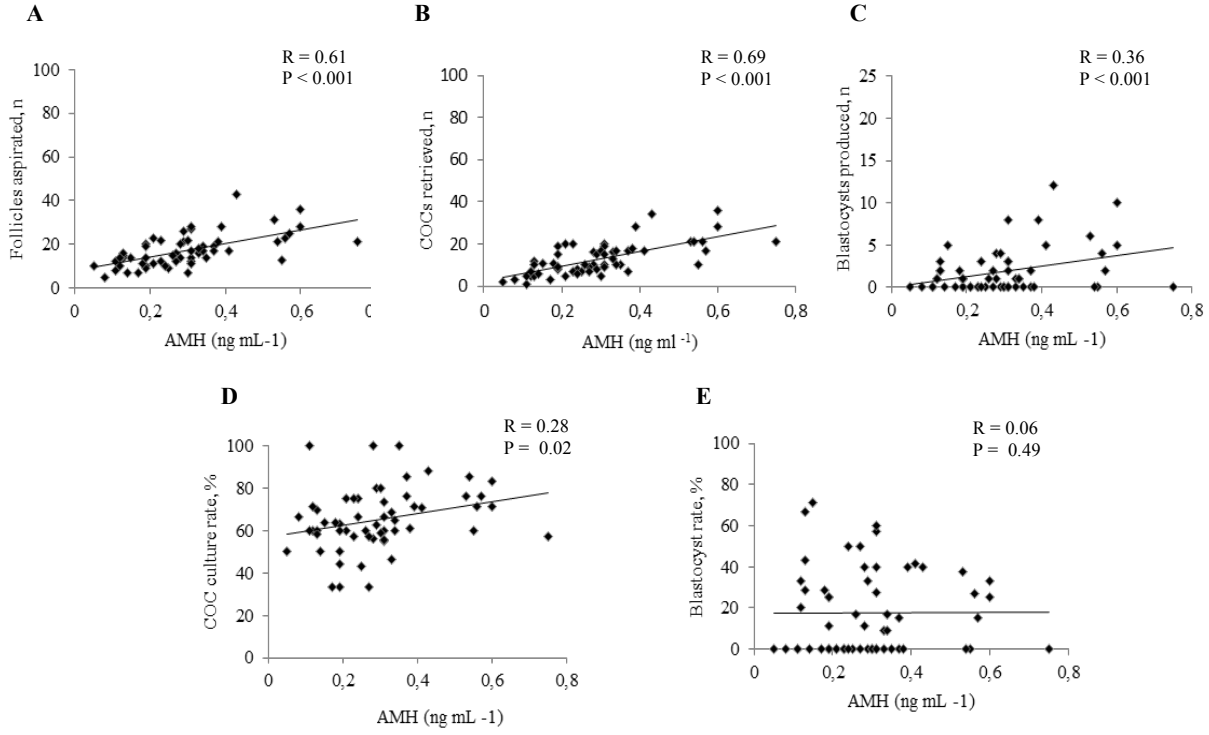

Figure 5. Correlation between plasma AMH concentrations and variables related to ovum pick-up (OPU) and *in vitro* embryo production (IVP) in *Bos taurus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D) and the blastocyst rate (%, E) and the plasma anti-Mullerian hormone (AMH) concentration in Holstein (*Bos taurus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. Adapted from Guerreiro *et al*. (2014).

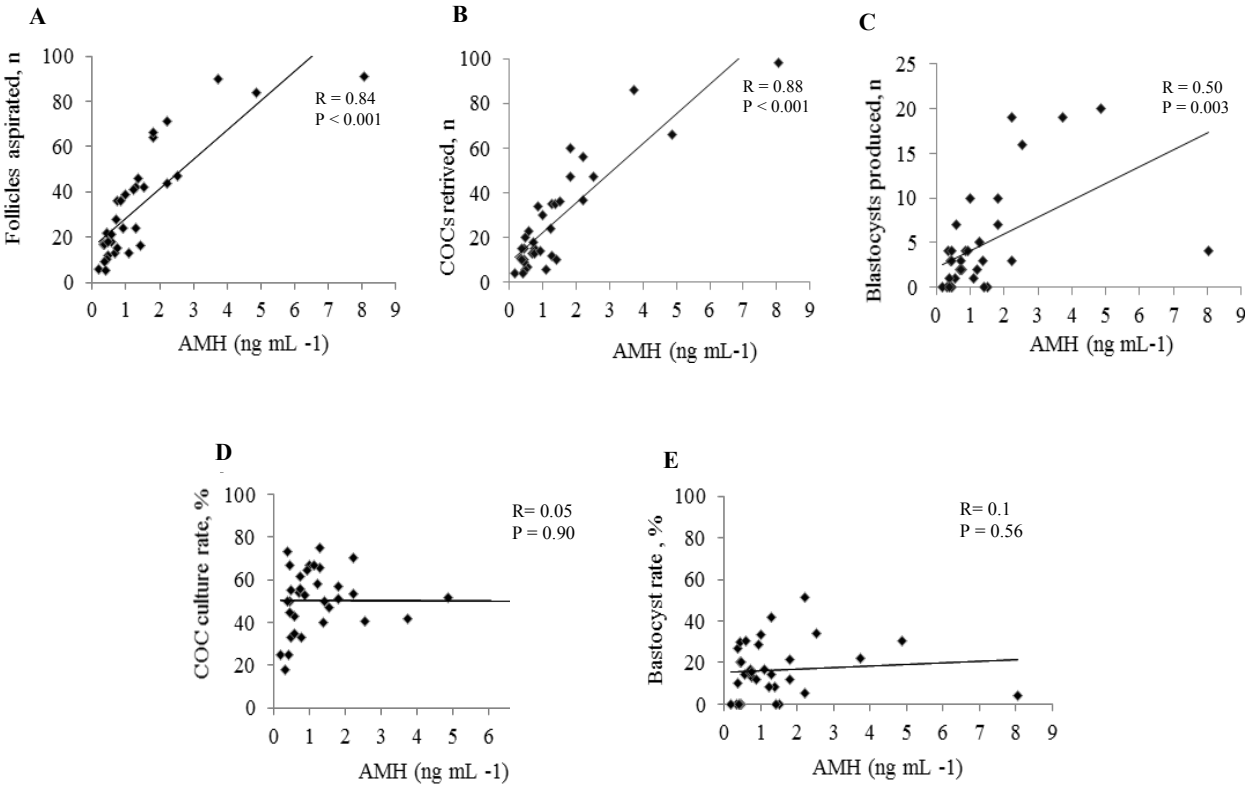

Figure 6. Correlation between plasma AMH concentrations and variables related to ovum pick-up (OPU) and *in vitro* embryo production (IVP) in *Bos indicus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D) and the blastocyst rate (%, E) and the plasma anti-Mullerian hormone (AMH) concentration in Nelore (*Bos indicus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. Adapted from Guerreiro *et al*. (2014).

Table 1. Plasma AMH concentrations and embryo results (mean ± SE) after OPU-IVP in *Bos taurus* (Holstein) and *Bos indicus* (Nelore) donors classified into two AMH categories.

	Plasma AMH concentration		P value
	Low	High	
Bos taurus			
Number of animals	32	27	-
Plasma AMH (ng/ml)	0.2 ± 0.01	0.4 ± 0.02	<0.0001
Cleavage rate (%)	54.5 ± 5.6	58.6 ± 5.3	0.98
Blastocysts produced per OPU	1.2 ± 0.3	3.0 ± 0.7	0.04
Blastocyst rate (%)	19.8 ± 4.2	20.6 ± 4.0	0.60
Bos indicus			
Number of animals	18	16	-
Plasma AMH (ng/ml)	0.5 ± 0.05	2.0 ± 0.3	<0.0001
Cleavage rate (%)	76.0 ± 8.2	89.8 ± 4.0	0.14
Blastocysts produced per OPU	2.2 ± 0.5	7.0 ± 1.7	0.0067
Blastocyst rate (%)	27.4 ± 5.5	33.7 ± 6.5	0.41

Adapted from Guerreiro *et al*. (2014).

AMH and fertility

Recent reports observed a positive association between AMH and fertility in dairy cows (Ribeiro *et al*., 2014; Jimenez-Krassel *et al*., 2015). For example, a large study done in Florida, USA reported that cows with low AMH concentrations had lower pregnancy results following first service and greater incidence of pregnancy loss between day 30 and 65 of gestation (Ribeiro *et al*., 2014). Moreover, dairy cows with relatively low circulating AMH concentrations as heifers also had the lowest survival rate after freshening

for the first time compared with age-matched herdmates having greater AMH concentrations (Jimenez-Krassel *et al*., 2015). Interestingly though it appears that the type of AI may determine the value of using AMH in a breeding program. For example, an elegant study done by Ribeiro *et al*. (2014) showed positive association between circulating AMH and P/AI in cows inseminated after estrus detection. However, no association between circulating AMH and P/AI was observed when cows had their ovulation synchronized for timed AI (TAI; Ribeiro *et al*., 2014). Thus, it appears that the use of TAI protocols may override possible associations of AMH with field fertility and that may help explain some of contrasting results we have recently observed when working with Nelore cows. For instance, accordingly with other study (Santos *et al*., 2014), we have found no correlation of a greater antral follicular population (animals likely having greater AMH) and conception results following timed AI protocols in mature Nelore cows (n = 758) or heifers (n = 1,113, Table 2; Baruselli *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data). Additionally, although antral follicle population has been associated with embryo production, no effect of donor antral follicle population was observed on pregnancy establishment after transferring the produced embryos (Fig. 7; Bragança *et al*., 2014; Fig. 8; Guerreiro *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data).

Table 2. Number of animals enrolled in the trial, body condition score (BCS), age (months), ovulation and/or pregnancy rate after TAI in Nelore heifers or cows according to the antral follicle population (AFP; measured at day 4 of TAI protocol; the expected moment of wave emergence) category in which animals were assigned. Data presented as percentage or average ± standard error of the mean (SEM).

	Antral follicle population categories			Total	P value
	Low	Medium	High		
Cows					
Number of animals	255	250	253	758	-
BCS (1 - 5)	3.00 ± 0.02	3.02 ± 0.02	3.02 ± 0.02	3.01 ± 0.01	0.29
Antral follicle population (n)	24.5 ± 0.5^{C}	39.2 ± 0.9^{B}	56.3 ± 1.4^{A}	40.0 ± 0.7	<0.0001
Pregnancy rate (%)	47.1	53.6	45.5	48.7	0.89
Heifers					
Number of animals	371	371	371	1,113	-
Age, months	15.0 ± 0.1	14.9 ± 0.1	14.8 ± 0.1	14.9 ± 0.1	0.22
BCS (1 - 5)	3.27 ± 0.02	3.27 ± 0.02	3.31 ± 0.02	3.28 ± 0.01	0.74
Antral follicle population, n	7.1 ± 0.1^{C}	11.3 ± 0.1^{B}	17.2 ± 0.2^{A}	11.8 ± 0.2	<0.0001
Ovulation rate (%)	82.5	78.3	79.8	80.2	0.56
Pregnancy rate (%)	39.6	36.4	36.7	37.6	0.57

$^{A \neq B \neq C}$Data with different superscripts in the same line differ with P ≤ 0.05.

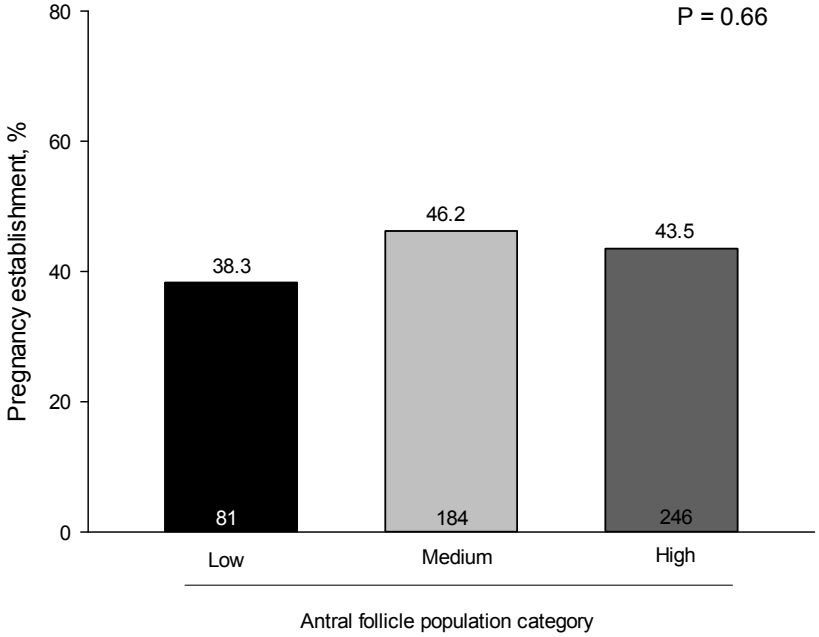

Figure 7. Pregnancy establishment in crossbred (*Bos indicus x Bos taurus)* recipients after embryo transfer of *in vitro* produced embryos according to the non-lactating Holstein donor antral follicle population category (low, medium or high). Adapted from Bragança *et al*. (2014).

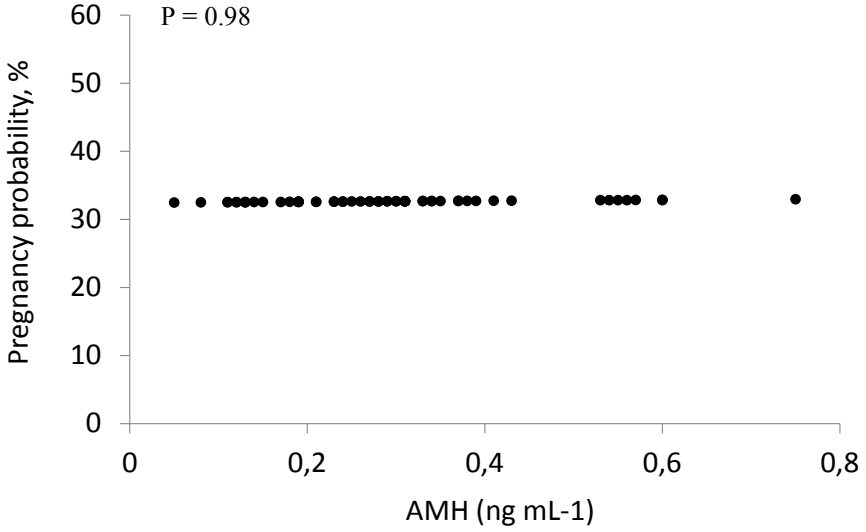

Figure 8. Relationship between circulating levels of AMH (ng/ml) and pregnancy probability in crossbred receptors after transferring *in vitro* produced embryos from Holstein donors (n = 107). Adapted from Guerreiro *et al*. (2015); USP/FMVZ, São Paulo, SP, Brazil; unpublished data.

Similarly, we failed to observe any association between circulating AMH and age at conception in Holstein heifers or interval from calving to conception in lactating Holstein cows (Carvalho *et al*., 2015; University of Wisconsin-Madison, Wisconsin, Madison, USA; unpublished data; Fig. 9). One important aspect to be discussed in our study though is that we have not taken into account heifers and/or cows that had been culled before pregnancy confirmation. This was a retrospective analysis rather than a manipulative or observational study.

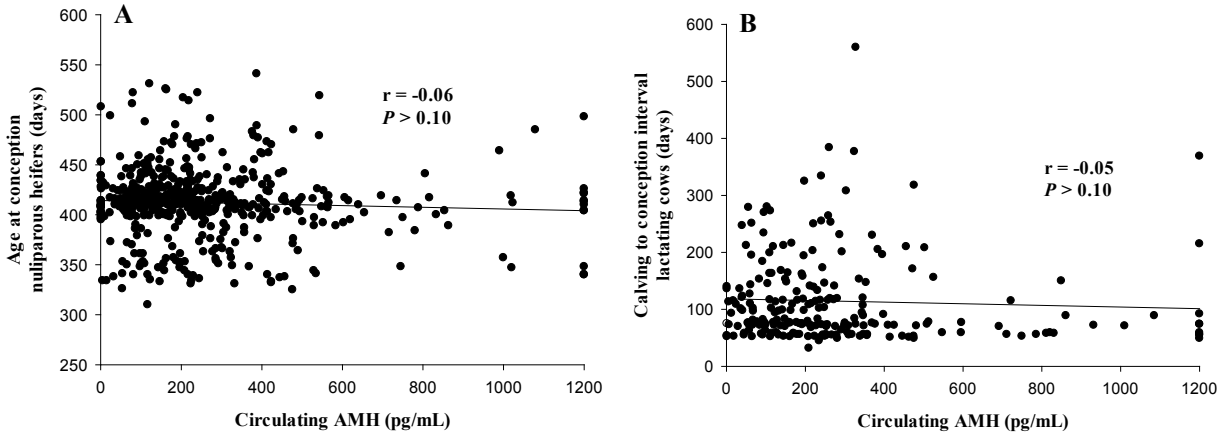

Figure 9. Relationship between circulating levels of AMH (pg/ml) and age (days) at conception in heifers (n = 528; panel A); and interval from calving-to-conception (days open) in lactating cows (n = 223; panel B).

Furthermore, because number of ovarian antral follicles appears to be correlated in cattle dam-daughter pairs (Walsh *et al*., 2014), and that may allow for selection of animals with greater AFP, we have recently looked into possible associations between circulating AMH in dam-daughter pairs in Holstein and Jersey breeds (Fig. 10; Batista *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data). The correlation in circulating AMH in dam-daughters although significant, was somewhat low (r = 0.18). Although, AFP in cattle is moderately heritable (0.31; (Walsh *et al*., 2014), epigenetic factors such as levels of negative energy balance during early fetal life (Evans *et al*., 2012) as well as dam-age and lactation status (Walsh *et al*., 2014) might likely influence antral follicle count in offspring. These epigenetic factors might then explain the poor correlation found in circulating AMH between dam-daughter pairs. Overall, the value of using AMH measurement to predict field fertility is still controversial and further studies using large numbers of animals are needed to draw final conclusions.

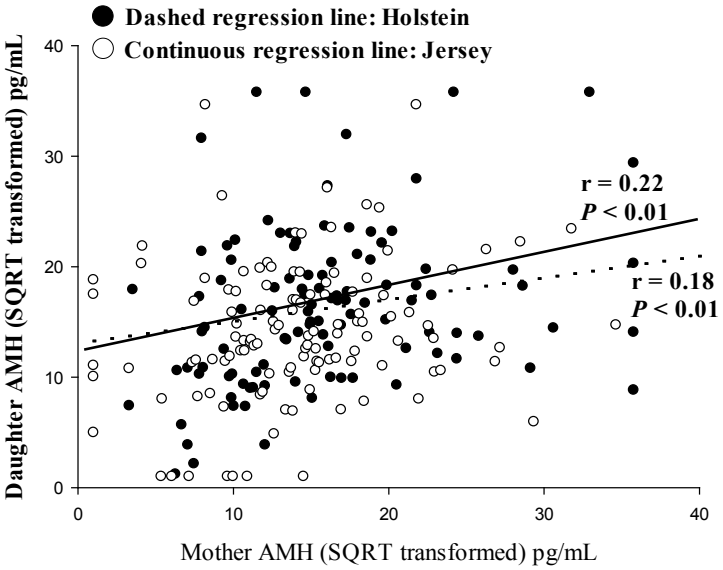

Figure 10. Correlation between circulating levels of AMH (pg/ml) in Holstein (n = 116) and Jersey (n = 106) dam-daughter pairs. AMH values are squared-root transformed. Data from Batista *et al*. (2015); USP/FMVZ, São Paulo, SP, Brazil; unpublished data.

Conclusions and future directions

Measuring circulating AMH in cattle can be useful to identify animals most likely to have improved superovulatory responses to gonadotropin treatment as well as best oocyte-donors for *in vitro* embryo production. In addition, although we cannot rule out the possibility of utilizing AMH to select cows with improved fertility; further basic and applied research are needed to elucidate whether AMH can be used by beef and dairy operations to identify animals with greater fertility and productive life.

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Estrus detection tools and their applicability in cattle: recent and perspectival situation

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Abstract

Good reproduction is key for successful dairy farming. Detection of estrus is the first step in getting a cow pregnant. Visual detection of estrus is a challenging job, to aid the farmer, estrus detection tools (EDT), such as pedometers, neck mounted collars to measure activity and pressure sensing devices to measure standing estrus, have been developed. EDT have proven useful in practical dairy farming, however, studies from the last five years reveal a great variation in sensitivity, specificity and positive predictive values. In research, the standard that is used to define a true estrus period can affect the performance of the EDT under investigation. Cow factors that can affect performance of EDT are number of ovulation after calving, milk production, lactation number, body condition score and lameness. The second step in getting a cow pregnant is insemination at the correct time. With EDT it is easier to determine optimal insemination time, which is 12 to 24 h before ovulation. The optimal time interval in which to inseminate seems to be about 5 to 17 h after an increase in activity as measured by pedometers or neck mounted collars. Novel measurements, such as rumination time, eating time, lying behavior, ultra-wide band technology to measure mounting and standing-to-be-mounted behavior and infrared thermography to measure temperature are being studied to further aid estrus detection.

Keywords: dairy cattle, estrus, insemination, technology.

Introduction

Good reproduction is key for successful dairy farming. Detection of estrus is the first step in getting a cow pregnant. Visual detection of estrus is a challenging job. The expression of standing estrus is only shown by about 50% of cows in estrus and lasts for a short period of time of about 5 to 7 h (Roelofs *et al*., 2005b; Sveberg *et al*., 2011). To aid farmers in detecting estrus and determining the optimal insemination time, many estrus detection tools (EDT) have been developed (reviewed by Roelofs *et al*., 2010; Saint-Dizier and Chastant-Maillard, 2012). For example, an increase in activity associated with estrus can be measured by pedometers or neck mounted collars and pressure sensing devices are on the market to detect cows expressing standing estrus.

A true estrus period can be detected by an EDT (true positive alert: TP) or not detected (false negative alert: FN). Outside a true estrus period, an EDT can give no alert (true negative alert: TN) or can give an alert (false positive alert: FP). To assess the performance of an EDT, sensitivity [TP/(TP+FN)], positive predictive value [TP/(TP+FP)] and specificity [TN/(TN+FP)] are often used (Roelofs *et al*., 2010). Rutten *et al*. (2014) concluded that an investment in activity meters for estrus detection is likely to be profitable for most dairy farms; however, this strongly depends on the increase in sensitivity that activity meters achieve, as compared with visual estrus detection. Although automated activity monitoring systems have proven useful as EDT in practical dairy farming (Michaelis *et al*., 2013; Neves and LeBlanc, 2015), studies from the last five years reveal a great variation in sensitivity and positive predictive values.

The second step in getting a cow pregnant is insemination at the correct time. The optimal time for insemination is 12 to 24 h before ovulation (Trimberger, 1948; Roelofs *et al*., 2006). Pedometers and neck mounted collars can be used to predict the time of ovulation (Roelofs *et al*., 2005a; Hockey *et al*., 2010) and therefore aid the farmer in deciding when to inseminate a cow.

In this review the performance and factors affecting the performance of different EDT will be discussed. Because this is elaborately reviewed by Saint-Dizier and Chastant-Maillard (2012), only studies performed over the last five years will be discussed in this review. The timing of insemination based on EDT and the effect on pregnancy rate will be discussed. New measurements that can aid in the detection of estrus will be reviewed.

Performance of EDT

Sensitivity and positive predictive value (PPV) varies between studies and EDT. In Table 1 the performance of different EDT is presented. Sensitivity ranged from 36 to 78% and is in all studies greater than the sensitivity of visual observations (range: 20 to 59%). PPV ranged from 74 to 97% and is not consistently better or worse thanvisual observations (Palmer *et al*., 2010; Holman *et al*., 2011; Michaelis *et al*., 2014). When pedometers were compared with neck mounted collars,

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sensitivity was greater but PPV was less for pedometers (Holman *et al*., 2011; Chanvallon *et al*., 2014). A better sensitivity means less false negative alerts, resulting in more detected true estrus periods. A better PPV means less false positive alerts, so less alerts are given when a cow is not in estrus. The design of the study can influence the number of false positive and false negative alerts. The number of FP and FN alerts as generated by the EDT depends on the definition of a true estrus period. Measurements of milk progesterone concentration are often used as golden standard for a true estrus period. Milk samples are collected 2 to 3 times weekly and a period of low progesterone, followed by a period of high progesterone, is considered to be a true estrus period. Based on individual progesterone profiles TP, FN and FP alerts from the EDT are assigned (Palmer *et al*., 2010; Holman *et al*., 2011; Kamphuis *et al*., 2012; Chanvallon *et al*., 2014). Using this definition for true estrus, cows that do not show any estrous behavior before ovulation (silent ovulation) will have more FN alerts resulting in lower sensitivities. This is not a malfunction of the EDT, but rather a physiological issue. Factors that can play a role in these FN alerts are discussed further on in this review. Another golden standard that is used for a true estrus period is the day of an insemination which led to a pregnancy (Jónsson *et al*., 2011). Using this definition silent ovulations do not generate a FN alert, because a cow is not inseminated when she is not detected in estrus at all. Sensitivity is likely to be greater when this golden standard is used. Michealis *et al*. (2014) used 21-day cow-periods according to the cycle length. After the voluntary waiting period, every cow started into a first 21-day cow-period and cows were observed for numerous cow-periods. When a cow was reported to be in estrus by visual observation or activity as measured by a neck mounted collar, estrus was confirmed by rectal palpation, ultrasonography and a blood sample for progesterone analysis. FN alerts were assigned when no alert was generated in a 21-day cow-period. Besides silent ovulations which generates a FN alert, the luteal phase can be prolonged or a cystic ovarian follicle can develop, which means that no ovulation occurs in a 21-day period (Lamming and Darwash, 1998). Therefore, an overestimation of FN alerts will probably occur by using 21-day cow-periods as golden standard. This might explain the low sensitivity found in the study of Michaelis *et al*. (2014) by neck mounted collars (36%).

Table 1. Sensitivity and positive predictive value (PPV) of different estrus detection tools (EDT).

References	EDT	Sensitivity %	PPV %	Housing	GS
Palmer *et al*., 2010	pressure sensing device	69	97	pasture	P4
	tail paint	65	94		
	VO[1] (3 times/day, 20 min.)	59	97		
Palmer *et al*., 2010	pressure sensing device	37	77	indoors	P4
	tail paint	26	92		
	VO[1] (3 times/day, 20 min.)	20	100		
Holman *et al*., 2011	neck mounted collar	59	94	indoors	P4
	pedometer	63	74		
	VO[2] (6 times/day, 10 min.)	57	93		
Kamphuis *et al*., 2012	neck mounted collar	78	78	pasture	
	tail paint	91	95		
					P4
Chanvallon *et al*., 2014	neck mounted collar	62	83	indoors	
	pedometer	71	71		
Michaelis *et al*., 2014	neck mounted collar	36	84	indoors	21dp
	VO[2] (2 times/day, 30 min.)	34	75		
Hockey *et al*., 2010	neck mounted collar	90	76	pasture	P4
Jónsson *et al*., 2011	pedometer	89	84	indoor	preg
Aungier *et al*., 2012	neck mounted collar	72	67	pasture	P4
Talukder *et al*., 2015	neck mounted collar	80	67	pasture	P4

VO = visual observation;[1]Visual observation of standing to be mounted;[2]Visual observation of vulva sniffing/being sniffed, chin-resting/being chin-rested on, mounting other cows and standing to be mounted, mucoid or bloody vaginal discharge; GS = golden standard for true estrus period; P4 = 2 or 3 times weekly milk sampling for progesterone concentrations; 21dp = 21-day cow-periods according to the cycle length; preg = confirmed pregnancy.

Factors influencing the performance of EDT

Different factors play a role in the number of false positive and false negative alerts generated by an EDT. It is clear that the threshold at which an estrus alert is generated by an EDT has a great impact on the sensitivity and PPV (Roelofs *et al*., 2005a; Hockey *et al*., 2010; Kamphuis *et al*., 2012). Physiological factors might also play a role in the performance of an EDT. Factors that decrease the expression of estrus will also decrease the sensitivity of an EDT when progesterone analyses are used as golden standard for a true estrus period. The first ovulation after calving is often not accompanied by an increase in activity or standing heat. Sensitivity of neck mounted collars for first ovulations after calving were found to be 23 and 30% in two studies (Aungier *et al*., 2012; Chanvallon *et al*., 2014). Sensitivity increased to 80% for second and later ovulations after calving. The same was found for estrus detection with pedometers (Chanvallon *et al*., 2014), where sensitivity for first ovulations after calving was 40% compared with 86% for subsequent ovulations. Ranasinghe *et al*. (2010) studied sensitivity of first, second, third and fourth ovulations after calving, which resulted in sensitivities of 45, 76, 79, and 89%, respectively. In normal, healthy cows, first ovulation occurs on average 28 days after calving (Johnson *et al*., 2012; Chanvallon *et al*., 2014). In practice, the voluntary waiting period is usually around 50 days. So, the low sensitivity of estrus detection found for first ovulations is not really an issue in practice. When however, many cows in a herd have an extended post partum anestrus, the performance of an EDT will be less. In interpreting and comparing research findings, it is important to take into account whether or not first ovulations were included in the calculations of sensitivity.

Lactation rank, milk protein content, body condition score, milk production, lameness and somatic cell count are studied for their effect on the performance of EDT. A high peak milk production as well as an above average daily milk yield and high production at the time of a preovulatory follicular phase were found to negatively affect sensitivity of neck mounted collars or pedometers. Sensitivity of neck mounted collars was 36% for cows with a peak milk production of more than 40 kg, whereas sensitivity was 68% for cows with a peak milk production of less than 35 kg (Chanvallon *et al*., 2014). Another study that investigated neck mounted collars and pedometers found a sensitivity of around 37% for cows with above average daily milk yield compared with around 60% sensitivity for all cows for both EDT (Holman *et al*., 2011). Aungier *et al*. (2012) concluded that if a cow was producing 10 kg less than another cow that was also in a preovulatory follicular phase, the odds of her preovulatory phase being detected by a neck mounted collar were greater by 67%.

Body condition scores of less than two resulted in a very low sensitivity for neck mounted collars (0%) and pedometers (20%; Holman *et al*., 2011). Only a few cows were in this category, so firm conclusion could not be drawn. Aungier *et al*. (2012) found that detection of a true estrus period by a neck mounted collar increased by a factor of 1.383 for each additional 0.25 BCS unit. No effect of somatic cell count (Holman *et al*., 2011; Aungier *et al*., 2012) on sensitivity of EDT was found. Milk protein content did (Talukder *et al*., 2015) or did not affect (Aungier *et al*., 2012; Chanvallon *et al*., 2014) sensitivity of EDT. Aungier *et al*. (2012) did not find a lower sensitivity in lame cows compared with non lame cows, whereas others did find a lower sensitivity in lame cows (Holman *et al*., 2011; Talukder *et al*., 2015). Lactation number did not affect sensitivity of neck mounted collars (Aungier *et al*., 2012) but did affect sensitivity of pedometers (Chanvallon *et al*., 2014). For cows in their first lactation, a higher sensitivity (77%) was found compared with cows with higher lactation number (52%). This is in agreement with the study of Roelofs *et al*. (2005a) in which cows in their first lactation had a longer duration of increase in activity and higher maximum steps during the increase in activity compared with cows with higher lactation number. Depending on the threshold calculation of an estrous related activity increase, a longer period of increased activity and more steps are more likely to give an alert.

Timing of insemination

To be able to give accurate insemination advice based on oestrus detection technologies, the parameters that are measured by the EDT to indicate onset of estrus should have a strong correlation with the time of ovulation and should be consistent between animals. A few studies have looked at the time of ovulation relative to the onset of estrus. The time of ovulation relative to the onset of estrus as measured by EDT is quite consistent between different studies. Intervals of 29.3 ± 3.9 h (n = 63 ovulations) and 30.2 ± 0.6 h (n = 20 ovulations) between the onset of oestrus based on pedometer measurements to ovulation were found in Holstein-Friesian and Japanese black cows, respectively (Roelofs *et al*., 2005a, Yoshioka *et al*., 2010). This agrees with the interval of 28.7 h (n = 60 ovulations) between onset of estrus based on neck mounted collars and ovulation in synchronized dairy cows (Valenza *et al*., 2012). An interval of 33.4 ± 12.4 h (n = 94 ovulations) was found between the onset of estrus as detected by neck collars and ovulation (Hockey *et al*., 2010). The interval between the first standing estrus as detected by a pressure sensing system and the time of ovulation was found to be 27.6 ± 5.4 h (n = 67 ovulations, Walker *et al*., 1996) and 29.0 ± 0.6 h in Japanese black cows (n = 20 ovulations; Yoshioka *et al*., 2010). The consistency in these intervals indicates that activity meters or pressure sensing systems can be

used to predict time of ovulation and advise on optimal time of insemination.

The optimal time of insemination relative to ovulation was found to be 24 to 12 h before ovulation (Trimberger, 1948; Pursley *et al*., 1998; Roelofs *et al*., 2006).

In 1948 the a.m. - p.m. guideline for time of insemination was established. This guideline recommends that cows observed in estrus in the morning should be inseminated in the afternoon, and cows observed in estrus during the afternoon should be inseminated the following morning (Trimberger, 1948). Since then several studies have examined the optimal time for insemination relative to the onset of estrus as detected by an EDT (Table 2). Combining the optimal time of insemination relative to ovulation, with the time of ovulation after detection of the onset of estrus, will give an optimal estrus to insemination interval. Roelofs *et al*. (2005a) calculated this interval to be 5 to 17 h after the onset of increased activity as measured by pedometers. This interval is comparable to the interval found in other studies on pedometers, electric pressure sensing systems or neck mounted collars (Table 2). However, the interval that Hockey *et al*. (2010) found is noticeably different. Even though they found about the same interval between onset of estrus and ovulation as in other studies, the optimal time for insemination relative to ovulation was much later (16-0 h before ovulation). This could explain why the optimal interval between onset of estrus and insemination is later. The reason for this discrepancy in optimal timing of insemination relative to ovulation is not clear.

Table 2. Optimal insemination intervals after onset of estrus as detected by different estrus detection tools (EDT).

References	EDT	Optimal insemination interval after onset of estrus (h)
Maatje *et al*., 1997	Pedometers	6 - 17
Roelofs *et al*., 2005	Pedometers	5 – 17
Yoshioka *et al*., 2010	Pedometers	10 -18
Stevenson *et al*., 2014	Neck mounted collars	13 -16[1] 9 -12[2]
Hockey et *al*., 2010	Neck mounted collars	24 - 40
Dransfield *et al*., 1998	Pressure sensing device	4 -12
Xu *et al*., 1998	Pressure sensing device	12 – 18
Dalton *et al*., 2001	Pressure sensing device	12

[1]Primiparous cows; [2]Multiparous cows.

Stevenson *et al*. (2014) found a difference in optimal interval for insemination relative to the onset of estrus based on neck mounted collars, between primiparous and multiparous cows. In primiparous cows, inseminations between 13 and 16 h after onset of estrus resulted in the highest conception rates, where as in multiparous cows insemination less than 12 h after onset of estrus resulted in the highest conception rates. Primiparous cows had a longer lasting increase in activity as measured by pedometers compared with multiparous cows, but the interval between onset of increased activity and ovulation was not different (Roelofs *et al*., 2005b). This would mean that in primiparous cows, the interval in which an insemination results in comparable conception rates is larger than in multiparous cows. Consequently, insemination shortly before ovulation does not compromise conception rates in primiparous cows as it does in multiparous cows. A possible explanation could be a difference in quality and thereby the fertile lifespan of an oocyte. Primiparous cows have lower NEFA concentration after calving compared with multiparous cows (Wathes *et al*., 2007). Elevated NEFA exposure can compromise follicle growth and result in inferior quality oocytes (Van Hoeck *et al*., 2014). When the fertile lifespan of the oocyte is compromised, it is more important to have the sperm at the site of fertilisation ready when ovulation occurs. The difference in optimal insemination interval between primi- and multiparous cows is worth further investigation.

A study with sex-sorted semen in dairy heifers resulted in the highest conception rates for inseminations performed between 20 and 24 h after the onset of estrus as detected by a pressure sensing device (Sá Filho *et al*., 2010). Further research on optimal insemination intervals for heifers as well as for the use of sex-sorted semen is needed to optimise reproductive efficiency on dairy farms.

New measurements for estrus detection

Research on increased activity associated with estrus has already been performed more than 60 years ago (Farris, 1954). In the last five years, other measurements to aid in estrus detection have been studied. Among these new measurements are lying, eating and ruminating behaviour, feed intake, water intake, temperature measurements, body weight, sound and motion measurements. Jónsson *et al*. (2011) automatically recorded lying behavior as well as number of steps. True estrus periods (n = 18) were defined as periods around inseminations that led to confirmed pregnancy. Sensitivity was 50% when only lying behavior was used to detect estrus. A combination of number of steps and lying behavior, did not result in a higher sensitivity than using number of steps alone

(89%). Specificity was high for the number of steps (99.4%), lying behaviour (99.6%) and the combination (99.8%). PPV increased by 10% when lying behaviour was combined with the number of steps, so less false positive alerts were generated compared to using number of steps alone. Silper *et al*. (2015) studied lying and standing behaviour in heifers. An increase in activity (as measured by the number of steps) combined with ovarian ultrasonography was used to define a true estrus period. Both lying and standing behaviour differed on the day of estrus compared with non-estrus days. A large variation was found between heifers in both standing and lying measurements. Especially the length of the longest standing bout and its relationship with the time of onset estrus (as measured by increased number of steps) seems a promising aid in estrus detection. Measurements of lying behavior, standing behavior and number of steps can be combined in a sensor. The combination of these measurements is likely to result in less false positive alerts than measurement of increase in number of steps alone. This can lead to less inseminations performed on cows not in estrus.

Changes in rumination time around estrus have been studied in the last few years (Reith and Hoy, 2012; Reith *et al*., 2014a; Talukder *et al*., 2014; Pahl *et al*., 2015). One study found that measurement of rumination time alone or the combination of rumination time and activity did not result in a more accurate estrus detection performance than activity alone (Talukder *et al*., 2014). This finding does not agree with other studies that found that measurements of rumination time could aid in estrus detection (Reith and Hoy, 2012; Reith *et al*., 2014a; Pahl *et al*., 2015). In those studies rumination time was reduced by an average 20%, on the day before insemination (Pahl *et al*., 2015) or on the day of estrus as defined by activity measurements or visual observation (Reith and Hoy, 2012; Reith *et al*., 2014a). A >10% decrease in rumination time on the estrus day was found in more than 70% of the cows, whereas about 6% of the cows showed an increased rumination time on the estrus day. A high variation in the decrease of rumination time was found (Reith and Hoy, 2012). Feeding time and roughage intake decreased around estrus with approximately 20 and 10%, respectively (Reith *et al*., 2014b; Halli *et al*., 2015; Pahl *et al*., 2015). Concentrate intake was not affected by estrus (Reith *et al*., 2014b). Rumination time and eating time can be measured automatically by neck mounted collars (e.g. SCR heatime, Nedapsmarttag neck), but individual roughage intake is difficult to measure in practice. Therefore, measurements of rumination and eating time to aid in estrus detection are promising. More research on the factors affecting rumination and eating time and sensitivity, PPV and specificity, however, is needed.

Vaginal temperature increases before ovulation (Rajamahendran *et al*., 1989). Recently, studies were done to see whether infrared thermography could be used to detect estrus and predict time of ovulation. In one study, sensitivity of 75% was found with infrared thermography of the vulva and muzzle every four hours. This sensitivity was higher than the sensitivity with six times daily visual observations (67%). Specificity and PPV, however, were lower with infrared thermography (57 and 69%, respectively) compared with visual observations (86 and 89% respectively, Talukder *et al*., 2014). A study done by the same group in which eye, vulva and muzzle temperature were measured using infrared technology showed poor performance for detecting estrus (Talukder *et al*., 2015).

A novel approach to detect estrus is the use of ultra-wide band technology (UWB). This technology can measure 3-dimensional positioning and could be used to monitoring mounting and standing-to-be-mounted behavior. In a study, 9 out of 9 possible cows were detected in estrus automatically by UWB technology and 6 out of 6 cows were correctly identified as not in estrus (Homer *et al*., 2013). Roelofs *et al*. (2005b) found that 90% of cows in estrus showed mounting behavior, whereas only 58% of cows in estrus showed standing-to-be-mounted behavior. The first mount was displayed on average 29 h before ovulation. Automatic detection of mounting behavior could be a helpful tool in detection of estrus and determining optimal insemination time.

In conclusion

Performance of estrus detection tools varies between studies, but is overall better than visual observation of estrus. Taking into account factors that affect the performance of EDT such as first ovulations after calving, high milk production, lactation rank etc. and possibly adjusting the calculations for the threshold used to generate an alert might increase the performance of pedometers and neck mounted collars. Because the beginning of estrus is detected by the EDT, inseminations can be better timed, thus increasing conception rates. An interesting area of research is optimal insemination time in heifers, and when using sex-sorted semen. Other behavioural measurements, and measurements of physiological traits associated with estrus, are studied to aid in the detection of estrus and determining optimal insemination time. The combination of activity measurements and rumination, eating and lying time measurements seems promising.

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The developmental origins of health and disease: importance for animal production

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Abstract

The importance of management of pregnant animals during pregnancy has been long known to have effects on the quality of their offspring products. In this review, after defining and setting up the current understanding of the Developmental Origins of Health and Disease (DOHaD), effects on traits relevant to animal production, such as meat quality and lactation as well as general health are discussed, with a special interest for effects of periconceptional nutrition.

Keywords: animal production, DOHaD, fetal programming, lactation, meat.

Introduction

Animal management during pregnancy has been long known to have effects on the quality of offspring products. In this review, after explaining the current understanding of the Developmental Origins of Health and Disease (DOHaD), effects on traits relevant to animal production, such as meat quality and lactation as well as general health are discussed, with a particular interest for the effects of periconceptional nutrition. Since effects on reproductive function have been recently widely reviewed (Gardner *et al*., 2009; Chadio and Kotsampasi, 2014; Chavatte-Palmer *et al*., 2014; Kenyon and Blair, 2014), effects on offspring reproductive function have not been developed as the reader can refer to these reviews. Post-natal management will not be discussed thoroughly as this goes beyond the objectives of this review, but maternal environment impact on offspring's phenotype at adulthood will be.

Principles of the developmental origins of health and disease

In the 90's, Barker and co-workers' epidemiological studies underlined an increased risk of non-communicable metabolic diseases in people born Small-for-Gestational Age (SGA) and/or with a poor growth rate in infancy (Barker and Osmond, 1986; Hales *et al*., 1991; Barker, 1992). These observations, referred to as "Fetal programming", suggested that fetal and neonatal adaptations to a nutritionally poor environment induced permanent adaptations leading to a "thrifty phenotype", where the restricted individual favors energy storage and insulin resistance throughout his life. Such early and long-lasting adaptations increase the risk of developing metabolic pathologies at adulthood in the presence of excess food intake, often described as an "energy mismatch" between early life and adulthood (Hales and Barker, 1992, 2001). Subsequently, the concept of the "Predictive Adaptive Response" stated that cues about environment delivered during pregnancy to the developing organism could also induce adaptive responses that would favor long term survival in a similar environment but may be less favorable for survival in a different environment (Bateson *et al*., 2004). This plasticity could provide an evolutionary advantage in the case of environments that change over a few generations but be deleterious in case of even faster changes, when the environment is very different between fetal and post-natal life (Gluckman *et al*., 2009). Moreover, more recent focus on obesity has shown that excess maternal body weight and adiposity also induced fetal adaptations leading to adverse outcomes at adulthood that are curiously close to that observed with growth retardation.

It is now generally admitted that components of human obesity, type-2 diabetes (T2D) and hypertension, but also bone health (Goodfellow *et al*., 2010), psychiatric health (Khandaker *et al*., 2012) and fertility (Faure *et al*., 2015) take root during early development, throughout gestation and lactation, as stated in the "Developmental Origins of Health and Disease" (DOHaD) hypothesis (http://www.mrc.soton.ac.uk/dohad/). Indeed, many studies in humans and animals have demonstrated that an individual's nutritional and hormonal status during fetal development and early life plays an important role for his long-term control of energy metabolism (Barker, 1995; McMillen *et al*., 2008). Epidemiological and experimental reports indicate that epigenetic mechanisms are the link between early life events and health later in life, with epigenetic marks being considered as long-lasting environmental cues (Gabory *et al*., 2011). Animals are affected by this process, which can also affect traits related to production, such as lactation, meat quality and other production traits (Wu *et al*., 2006; Kenyon and Blair, 2014; Fig. 1).

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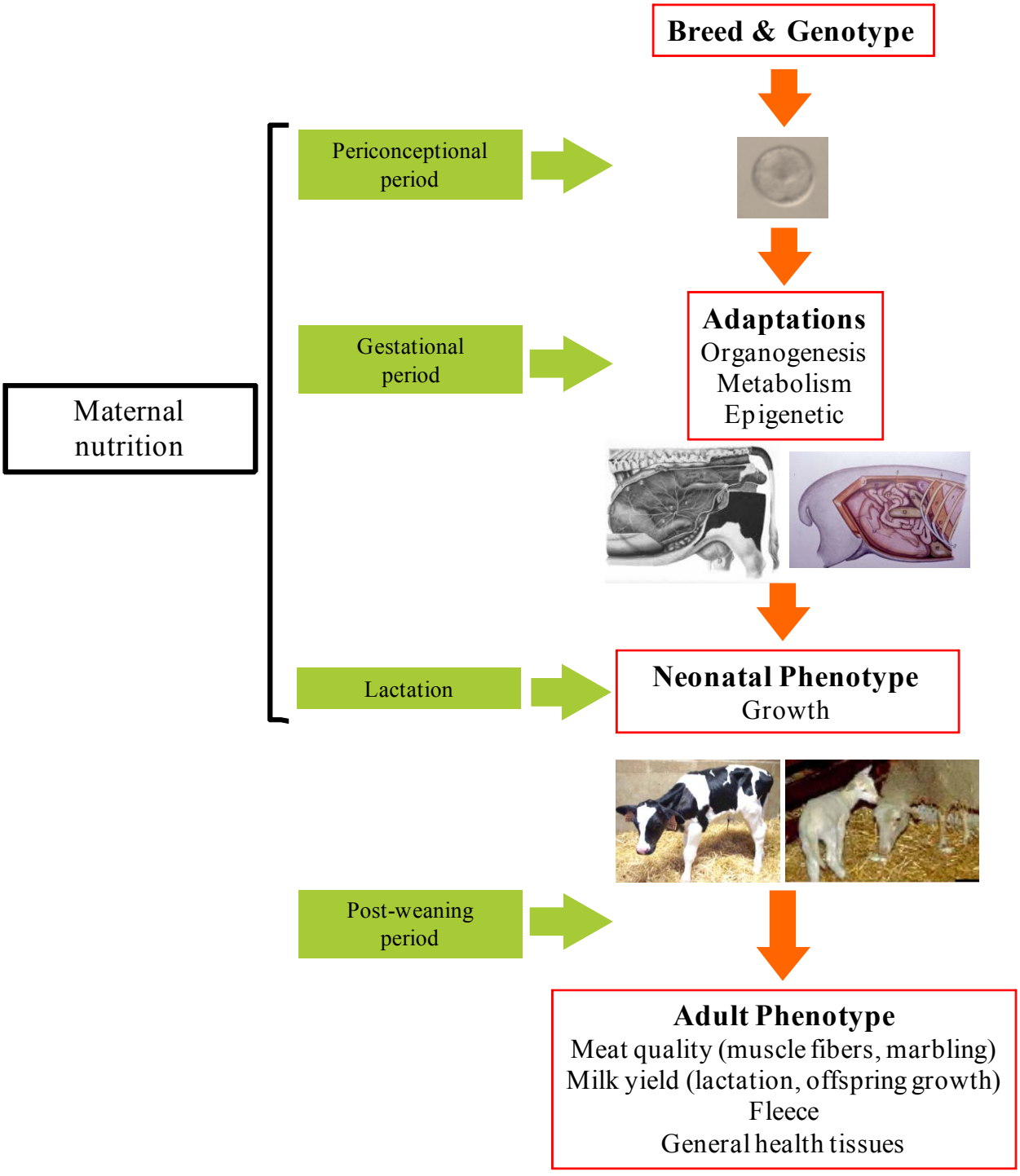

Figure 1. Principles of the developmental origins of health and disease.

DOHaD and the quality of products

It is only recently that studies have started to explore the effects of maternal nutrition during pregnancy on livestock performance in mammals. Although considerable effort has been directed towards defining nutrient requirements of animals over the past 30 years, suboptimal nutrition during gestation remains a significant problem for many farm animal species as well worldwide (including cattle, pigs, and sheep; Wu *et al.*, 2004). Ruminants have been the main focus of research, but data in pigs, rabbits and horses also exist. Major observed effects, as detailed below, are summarized in Fig. 2.

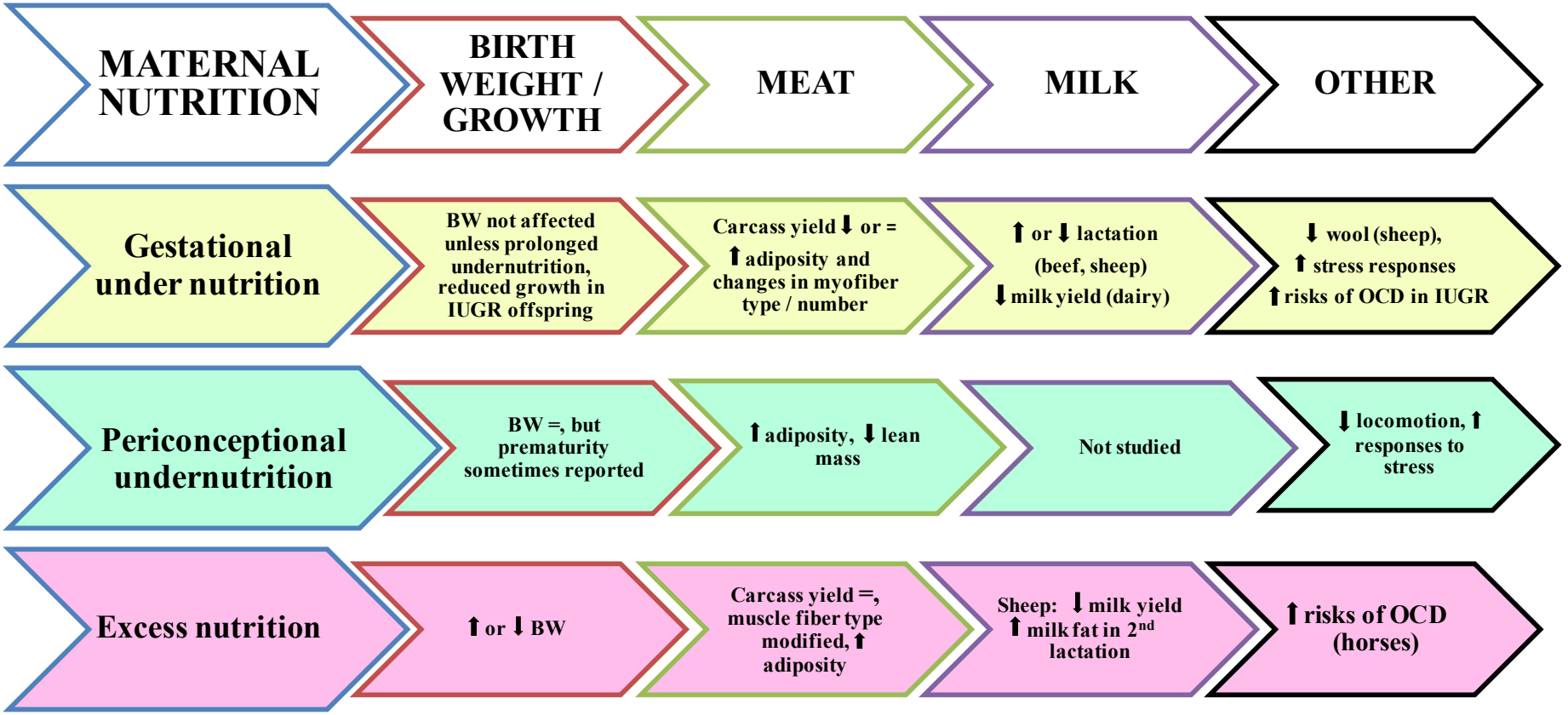

Figure 2. Main effects observed on production traits after maternal undernutrition or overnutrition during gestation, or after periconceptional undernutrition. BW = birthweight; IUGR = intra-uterine growth retardation; OCD = osteochondrosis.

Growth

Growth and attainment of adult size is essential for production. Adult size is dependent on the genetics of the individual, but will be modulated by nutrition and environmental parameters at large. Already in the 1970's, McCance and Widdowson demonstrated that "critical periods" of undernutrition in the prenatal or immediate post-natal period can lead to growth retardation that cannot be recovered by subsequent catch-up growth (McCance, 1976). More studies using animal models have shown how intra-uterine growth restriction and post-natal under-nutrition can restrict offspring size as adults (Desai *et al*., 2005; Bieswal *et al*., 2006). In ruminants, undernutrition during the first half of gestation, although birth weight was usually not affected, was shown to impact metabolic function of sheep and cattle offspring, resulting in altered production and body composition later in life (Ford *et al*., 2007; Long *et al*., 2010, 2012). Undernutrition of ~75% of recommended allowance during early stages of pregnancy compromises placental angiogenesis, cotyledon weight, and thus, fetal development (Zhu *et al*., 2007), with significant impact on development and function of liver and pancreas (Symonds *et al*., 2010). Overnutrition can also restrict placental and fetal development, resulting in decreased birth weights, post-natal growth, and altered body composition (Caton *et al*., 2007). Protein imbalance, with dams either nutrient restricted or supplemented with proteins, affects the development of the fetus through gestation, driving to born-too-thin or overweight neonates, respectively, both in bovine and ovine models (Funston *et al*., 2010).

Beef cattle - In order to reduce fetal growth, severe maternal undernutrition is required for at least a third or half of pregnancy in beef cattle (Greenwood and Cafe, 2007; Martin *et al*., 2007). Fetal growth restriction, however, may also result from twinning, excess heat and is observed in heifers as opposed to multiparous animals (Greenwood and Cafe, 2007). Intra-uterine growth retardation (IUGR) due to maternal food restriction throughout gestation (difference of 10 kg at birth) was shown to lead to reduced post-natal growth (Greenwood and Cafe, 2007). Moreover, undernutrition during the last third of pregnancy will decrease birth weights with a potential negative impact on long-term growth and body composition of the progeny (Underwood *et al*., 2010).

Dairy cattle - While growth (2 years old cows) and milk production of the dam have clear implications for development of the fetus (Banos *et al*., 2007), diet during pregnancy is the primary modifiable factor that has a substantial influence on body condition and energy status of the dairy cattle as well as viability and body composition of newborn calves. Like in beef cattle, reduced post-natal growth was observed in naturally occurring low birth weight Holstein calves (Swali and Wathes, 2006).

Sheep - Effects of maternal dietary level and timing in sheep have been recently very thoroughly reviewed (Kenyon and Blair, 2014). Like in cattle, maternal undernutrition generally does not reduce birth weight, although offspring may be lighter at weaning, in particular when undernutrition has been prolonged until term (Kelly *et al*., 1996, 2006; Field *et al*., 2015). Variation in the effects of undernutrition may also depend on the breed, or genetic / epigenetic background. Indeed, maternal undernutrition from 28 et 78 days of pregnancy did not reduce fetal weight in "Baggs" ewes which originated form a nomadic herd living in harsh conditions, whereas reduced fetal weight was observed in ewes bred for several generations in good conditions at the University of Wyoming (Vonnahme *et al*., 2006). These differences were accompanied with different placental responses to undernutrition, with earlier placental maturation in the "Baggs" ewes (Vonnahme *et al*., 2006). Heat stress during pregnancy also induces IUGR in sheep (Barry *et al*., 2008). In the field, the combination of maternal heat stress and undernutrition during pregnancy reduces lamb birth weight in ewes, which seem to better adapt to restricted feeding than to thermal stress (Sejian *et al*., 2011).

Overfeeding may also be harmful. As a result of dietary induced obesity in ewes, fetuses at mid-gestation were macrosomic, hyperglycemic, hyperinsulinemic with markedly increased pancreatic weight and β-cell numbers compared with fetuses of ewes fed to requirements (MacLaughlin *et al*., 2005; George *et al*., 2010; Zhang *et al*., 2011a). Although they were still hyperglycemic at birth, the insulin secretion was blunted with decreased pancreatic growth and β-cell numbers by the end of gestation (Zhang *et al*., 2011a).

Horse - In the horse, fetal growth is limited mainly by the uterine size of the dam, as shown by experiments using artificial insemination or embryo transfer in horses of different size (Walton and Hammond, 1938; Tischner, 2000; Allen *et al*., 2002, 2004; Peugnet *et al*., 2014, 2015b). These works have demonstrated the importance of fetal growth for the achievement of the expected adult size, with growth retarded foals being permanently affected in terms of growth, at least for some bone segments. This is of particular importance in these species, where reduced adult size will limit access to studbooks and to some sporting events, and may affect performance. Similar experiments in sheep indicate that these effects on birth weight are not driven by changes in maternal insulin sensitivity during pregnancy (Oliver *et al*., 2015).

In the horse, similar to what was described in sheep, maternal overnutrition is reported to reduce glucose uptake and reduce growth rate of two thirds of pregnancy (Satterfield *et al*., 2010). In equidae, however, maternal undernutrition does not seem to affect birthweight nor subsequent growth, at least until

weaning (Wilsher and Allen, 2006; Ousey *et al*., 2008; Peugnet *et al*., 2015a), which does not preclude effects on performance (Rossdale and Ousey, 2003).

Meat quality

Meat quantity depends on the muscle mass whereas meat quality will depend on muscle fibers, which play an important role for tenderness (Guillemin *et al*., 2009) and the intramuscular fat, which is a major component of flavor. Different levels of intramuscular fat will be looked for according to cultures and cooking traditions.

The fetal period is crucial for skeletal muscle development, because no net increase in the number of muscle fibers occurs after birth in farm animals (Greenwood *et al*., 2000; Nissen *et al*., 2003). Myogenesis takes place during several chronologically distinct phases occurring in fetal life: proliferation of precursor cells (myoblasts), fusion of myoblasts into differentiated multinuclear cells (myotubes), and differentiation of these cells into muscle fibers. Myotube formation gives rise to primary and secondary muscle fibers, respectively. Muscle fiber generations are set up around the last third of gestation in larger species - day 180 in cattle (term = 280-284 days), around 90 days in pigs (term = 115 days) and in the early post-natal period in less mature species such as rabbits (first month after birth) and there is no increase in muscle fibers thereafter (Stickland, 1978; Picard *et al*., 2002; Oksbjerg *et al*., 2004). Therefore, a reduction in the number of muscle fibers during fetal development will lead to reduced muscle mass in the adult. Fiber type is also important for production: slow oxidative fibers (Type I) originate from primary muscle fibers, and fast, type II myofibers, which mostly develop from secondary fibers, mature into type IIA (fast-oxidative glycolytic) or IIB (fast-glycolytic) fibers. Type II fibers are more efficient in terms of growth (Du *et al*., 2010), but oxidative, and more so, slow oxidative fibers, are generally more likely to produce more tender meat, although there are clear discrepancies between different muscles (Guillemin *et al*., 2009).

Intramuscular fat (marbling) is dependent on the presence of intramuscular adipocytes. Adipocytes originate primarily from mesenchymal cells in fetal life around mid-gestation at the same time when secondary muscle fibers are produced (Du *et al*., 2010), but ontogenesis still needs to be completely elucidated (Boone *et al*., 2000; Hocquette *et al*., 2010), although intramuscular adipogenesis can be modified through maternal nutrition during pregnancy in sheep (Tong *et al*., 2008). Triglycerides are initially stored within the muscle fibers and then, in the immediate postnatal period, intramuscular adipocytes will increase in size and volume (Hocquette *et al*., 2010). The hyperplasia of adipocytes is an important factor for marbling during growth (Hocquette *et al*., 2010).

Maternal undernutrition

Beef cattle - Severe maternal undernutrition leading to fetal growth restriction in beef cattle significantly reduces postnatal growth compared to controls but was not shown to affect muscle fiber characteristics, carcass composition and retail yield at similar carcass weight (Greenwood and Cafe, 2007). In regions where droughts are common, however, cows may experience periods of undernutrition during pregnancy, which may affect meat quality. Traditionally, they are supplemented in late gestation but not earlier (Du *et al*., 2010). In these harsh conditions, protein supplementation of pregnant cows in mid-gestation (day 60 to 180), or improvement of the pastures through irrigation, however, was shown to increase lean growth and reduce fat in offspring (Du *et al*., 2010). In a study using Angus x Gelbvieh cattle, Long *et al*. (2012) found no effect of maternal global undernutrition with or without protein supplementation during the first half of pregnancy on body weight or organ weight at slaughter (around 1.5 years of age). The ratio of semitendinosus muscle to carcass weight, however, tended to be reduced in the offspring from non-supplemented undernourished dams, with average adipocyte size increased in several anatomical locations (Long *et al*., 2012). The effect of increased or reduced maternal protein intake on offspring skeletal muscle development, however, is dependent on the timing of the supplementation during pregnancy as well as the sex of the offspring, as demonstrated in beef heifer offspring (Micke *et al*., 2011): as adults, males born to heifers fed a low protein diet during the first trimester of pregnancy and subsequently a high protein diet had greater *longissimus dorsi* muscle cross-sectional area compared to those whose dams were exposed to continuous high protein diet, whereas there was no effect of maternal protein intake on the female offspring. In contrast, maternal protein restriction in the second trimester was associated with higher *longissimus dorsi* muscle cross-sectional area in male and female offspring (Micke *et al*., 2011) although there was no effect on the *longissimus dorsi* weight/carcass weight ratio (Micke *et al*., 2010). These changes were associated with changes in the muscle expression of insuline-like growth factors IGF1 and IGF2 and their receptors (Micke *et al*., 2010, 2011).

Dairy cattle – In dairy cattle, conception usually takes place during early lactation, and thus lactating dams are in relative energy deficit compared to non-lactating dams (Funston and Summers, 2013). In a retrospective study of more than 1500 dairy calves, the size of the dam and its milk yield were shown to be the two most important factors influencing birth weight, with lower birth weights of calves born to high yielding cows with similar body size (Kamal *et al*., 2014). When heifers and cows were compared in a small number of Holstein females, maternal parity did not affect girth,

birthweight nor glucose metabolism in the first month post-partum, although the birthweight and withers height of first born calves were reduced (Bossaert *et al*., 2014).

Sheep - In sheep, several studies indicate that maternal undernutrition in the early stages of pregnancy can affect meat quality, although birth weight is not always affected (Kenyon and Blair, 2014). Indeed, an extended period of maternal nutrient restriction during the first half of gestation results in relatively normal birth weights, but leads to increases in the length and thinness of the neonates, increased adiposity, and suppressed glucose tolerance (Whorwood *et al*., 2001; Ford *et al*., 2007). Moreover, carcass weight quality appears to be affected more by genetic background and litter size at birth than by maternal nutrition, except when undernutrition lasts most of the gestation (Kenyon and Blair, 2014). Indeed, the placenta adapts to the nutritional environment to minimize nutritional consequences to the fetus, although the extent of its adaptive capacities depend on the timing and intensity of the nutritional insult (Fowden *et al*., 2008; Symonds *et al*., 2012).

Restricted fetal nutrition throughout gestation as experienced by ewes during twin pregnancies leads to increased fetal adiposity (Edwards *et al*., 2005). Undernutrition of twin-bearing ewes throughout pregnancy led to low-birthweight lambs that remained smaller until adolescence and had poor energetic efficiency (Husted *et al*., 2007). Moreover, offspring were shown to have disturbed responses to fasting at 6 months of age in terms of leptin (reduced), IGF1 and cortisol (increased; Kongsted *et al*., 2013). When low (mean 2.29 kg) and high (mean 4.84 kg) birthweight lambs were compared, although the weight of the *semitendinosus* muscle was very significantly decreased in low birthweight lambs, the number of myofibers was similar but the fact that low birthweight lambs did not catch up in muscle growth may be due to the reduced myonuclei number (Greenwood *et al*., 1998, 2000).

Like in cattle, however, the timing of undernutrition matters and numerous nutritional planes have been studied, using animals of different breeds, making comparisons rather difficult (Kenyon and Blair, 2014). If most undernutrition studies do not indicate a strong effect on post-weaning liveweight, higher adiposity has been sometimes observed (Kenyon and Blair, 2014). In ewes undernourished in early gestation, term fetuses had more adipose tissue compared to controls that were fed *ad libitum* (Bispham *et al*., 2003), which is associated with increased glucocorticoid sensitivity (Gnanalingham *et al*., 2005; Mostyn and Symonds, 2009). In another study, castrated males at 8 months of age born to white face dams that were nutritionally restricted to 50% of requirements from day 28 to 78 of pregnancy had heavier carcass weight than controls, but intra-abdominal fat deposits were also heavier and intramuscular triglyceride contents were increased as a result of the reduction of the activity of carnitine palmitoyltransferase-1, which is involved in fatty-acid oxidation (Zhu *et al*., 2006; Ford *et al*., 2007). Moreover, the total number of muscle myofibers was decreased with an increased ratio of IIB fibers (fast-glycolytic fibers) in muscle (Zhu *et al*., 2006). Similar findings were observed in another study where only twin animals were selected, with males and females analyzed separately (Daniel *et al*., 2007). This is an important point, as clear differences have been shown according to sex and litter size for many physiological parameters (MacLaughlin *et al*., 2010; Tarrade *et al*., 2015).

Pigs - In pigs, moderate IUGR is not always associated with a modification in carcass composition but very small piglets have slower growth and fatter carcasses than controls (Powell and Aberle, 1980; Mostyn and Symonds, 2009; Morise *et al*., 2011). Low birthweight piglets are also characterized by a reduced number of enlarged myofibers (Rehfeldt and Kuhn, 2006). Maternal undernutrition of Large White sows from mating to 50 days of pregnancy did not affect carcass weight, lean tissue and adipose tissue yield in offspring, whereas the composition of muscle in terms of myofiber types was slightly affected, with a reduced percentage of type IIB fibers (Bee, 2004). Maternal protein restriction, however, was shown to reduce the lean and increase the fat contents of offspring at 6 months of age with a tendency for reduced number of muscle myofibers associated with reduced expression of IGF2 mRNA (Rehfeldt *et al*., 2012).

Iberian pigs, which are genetically different form modern commercial pigs, deposit more intramuscular fat and are naturally leptin resistant (Ovilo *et al*., 2005; Munoz *et al*., 2009), presenting what resembles a "thrifty phenotype", which has been attributed to centuries of adaptation to low quality nutrition in semi-feral conditions (Lopez-Bote, 1998). In this breed that is prone to obesity, maternal undernutrition during pregnancy reduces birthweight and increases the incidence of IUGR piglets in the litter (Gonzalez-Bulnes *et al*., 2012). Female offspring appear to catch-up growth to controls at weaning whereas males are still growth-retarded at weaning (Gonzalez-Bulnes *et al*., 2012).

Excess maternal nutrition and obesity

Sheep - Extensive studies have shown that excess maternal nutrition retards placental and fetal growth, and increases fetal and neonatal mortality in sheep (Wallace *et al*., 2003). Excess maternal nutrition increases mid-gestation fetal weight (Ford *et al*., 2009). Lamb birth weight is similar to controls (Wallace *et al*., 2005; Zhu *et al*., 2009) or increased (Kenyon *et al*., 2011) according to the extent of the overfeeding and breed, but subsequent growth is similar (Kenyon *et al*., 2011). Maternal obesity, however, down-regulates

myogenesis through the Wnt/β-catenin signaling pathway (Tong *et al*., 2009).

Pigs - In pigs, excess maternal nutrition from mating to 50 days of pregnancy increased the adipose tissue yield in the offspring carcasses, without affecting overall carcass weight and lean yield, although muscle fiber type was modified (Bee, 2004). In another study, both reduced (50%) and excess (250%) maternal protein intake during pregnancy reduced piglet birthweight and birthweight/crown-rump length, reflecting adiposity (Rehfeldt *et al*., 2011). These effects, however, were not observed in fetuses at mid-pregnancy, indicating that placental insufficiency leading to IUGR had occurred in the second half of pregnancy (Rehfeldt *et al*., 2011). Offspring of dams fed a protein excess, however, did not differ from controls for muscle myofiber numbers and adipose tissue at 6 months of age (Rehfeldt *et al*., 2012).

In conclusion to this chapter, the favored fat development and reduced number in muscle myofibers mostly observed in maternal undernutrition experiments have been associated with changes in expression of insulin-like growth fators (IGFs; Micke *et al*., 2011), Growth hormone (Rehfeldt and Kuhn, 2006), transcription factors involved in adipogenesis such as the Peroxisome Proliferator-Activited receptor gamma (PPARγ; Tong *et al*., 2008, 2009) and nutrient sensors such as mTOR. Indeed, the main regulators of adipogenesis are the peroxisome proliferator activated receptor (PPARγ) and CCAAT -enhancer binding protein (C/EBP; Hausman *et al*., 2009). Moreover, fetal fat development may be favored by disturbed maternal plasma cortisol (reduced in undernourished pregnant sheep (Debus *et al*., 2012) or increased in pregnant sows fed a low protein diet (Otten *et al*., 2013)) observed during nutritional restrictions (Symonds *et al*., 2012).

Lactation

So far, the amount of data on the fetal programming of offspring lactation through the manipulation of maternal nutrition in domestic animals remains limited. Hence, in this part, the effects of under and over-nutrition are treated together. The organogenesis of the mammary gland begins early in pregnancy (Hovey *et al*., 2002; Houdebine, 2003) and may therefore be affected by maternal nutrition like other organs.

Beef cattle - In beef cattle, slower growing female calves whose dams have poor lactations tend to produce better lactation when they are adults and have offspring with faster growth, which in turn have reduced quality lactation as adults (Koch, 1972; Pala and McCraw, 2005).

Dairy cattle - In dairy cattle, the analysis of data available on the UK national fertility database and from Irish dairy cows showed that offspring from dams producing more milk before and during conception had reduced milk yields, increased somatic cell count and were culled earlier compared to those born to dams with lower milk yields (Banos *et al*., 2007; Berry *et al*., 2008). Similar observations were made in Spain, which showed that females born to dams that were lactating during early pregnancy produced significantly less milk compared to those born to dams that were not lactating and that this reduction in milk production was correlated to maternal production (Gonzalez-Recio *et al*., 2012).

Sheep - Kenyon and Blair (2014) have reviewed the effects of maternal nutrition on milk production in sheep. Maternal undernutrition from day 21 to 50 was shown to reduce the mammary gland weight in fetuses near term (Martín *et al*., 2012) and reduce milk production at first lactation (Paten *et al*., 2013). In contrast, fetal mammary duct density and fat production in milk in the second lactation was increased in female sheep born to dams that were fed *ad libitum* during pregnancy (1.5 times maintenance) compared to those born to control dams fed to maintenance (Blair *et al*., 2010). Nevertheless, the mammary mass was increased in the offspring of the maintenance group, and these offspring produced more protein and lactose, only in the first lactation at 2 years of age (Blair *et al*., 2010). The authors suggest that these effects limited to the first lactation may be the result of an "Adaptive Predictive Response", as defined above, where the "restricted" offspring would favor the survival of their own first offspring, with no investment in further lactations when the survival of the individual would be more hazardous. As a consequence, second generation effects were hence observed in two studies where grand-daughters of ewes fed a moderate diet during pregnancy were heavier at birth compared to the grand-daughters of dams that were fed *ad libitum* during pregnancy (van der Linden *et al*., 2009; Blair *et al*., 2010). Interestingly, this effect was confirmed in farmed minks bred for fur, when grand-mothers were protein restricted (Matthiesen *et al*., 2010).

Fleece

Fleece weight in sheep is affected by body size, which induces confounding factors for the analysis of maternal effects of this parameter and may explain why a reduction in fleece has been reported in response to maternal undernutrition (Schinckel and Short, 1961; Kenyon and Blair, 2014). One study reported a change in hair follicle number, however, which could persist throughout the life of the animal (Schinckel and Short, 1961).

General health issues

Thermogenesis - In the newborn sheep, brown adipose tissue (BAT, representing 1-2% of birthweight; Symonds and Lomax, 1992) is essential for ensuring effective adaptation to the extrauterine environment, in

particular thermogenesis. Neonatal pigs and horses, although they possess BAT (Ousey, 1997; Mostyn *et al*., 2014), are much more dependent on shivering thermogenesis to maintain heat production during cold exposure. Gestational BAT development depends on transplacental glucose supply to the fetus (Symonds *et al*., 2012). In contrast to rodents, brown fat, although present at birth, is very reduced in adult large animals as most BAT is progressively replaced by white fat (Symonds *et al*., 2012). Moreover, it may not have the same myoblastic origin as white fat (Budge *et al*., 2009).

Behavior – Few studies have focused on the impact of maternal nutrition on offspring behavior in large animals, although alterations in food intake and response to stress may be important to the breeding industry.

Food restriction in late gestation in sheep was shown to decrease voluntary milk intake in lambs from 3 to 60 days of age (Geraseev *et al*., 2006) whereas, in another study, feed intake was not affected after weaning (Sibbald and Davidson, 1998). In dairy goats, no effects were observed in feeding behavior and stress responses in male kids before weaning (Laporte-Broux *et al*., 2011). Although no effects on feeding behavior in females at one year of age, at 2 years of age, the cortisol response to ACTH injection was increased in offspring from restricted dams, suggesting a higher susceptibility to stress (Laporte-Broux *et al*., 2012). Similarly, in pigs born to dams fed a low protein diet during pregnancy, cortisol response to weaning was increased and the medulla area within the adrenal was increased (Otten *et al*., 2013).

Osteoarticular pathology - In horses, although maternal undernutrition does not affect birth weight, epidemiological and experimental data indicate that IUGR due to transfer of saddle embryos into ponies (Peugnet *et al*., 2014), but also feeding mares with concentrates during gestation (Van der Heyden *et al*., 2013; Peugnet *et al*., 2015a), may be associated with an increased risk of developing lesions of osteochondrosis in their foal, which is of strong economical importance for the horse industry. Since osteochondrosis is related to glucose/insulin metabolism, the effects may be linked to the observed trend for a reduced insulin sensitivity at 5 days of age in offspring of mares fed a high starch diet (George *et al*., 2009). More work is currently on-going in the authors' laboratory to explore this phenomenon.

Periconceptional programming

The developmental plasticity of embryos in the pre-implantation period leads to different embryo, feto-placental and post-natal responses to the environment (Laguna-Barraza *et al*., 2012). Specific targeting of the periconceptional period for experiments on maternal nutrition in large animals use different timing for the nutritional challenge, making it difficult to draw comparisons, although there are a lot of data available in model species and humans, which have been reviewed elsewhere (Watkins *et al*., 2010; Zhang *et al*., 2011b; Fleming *et al*., 2012; van Montfoort *et al*., 2012; Steegers-Theunissen *et al*., 2013; Lane *et al*., 2015). Experiments using embryo transfer between a nutritionally challenged oocyte or embryo donor have also provided valuable insight into periconceptional effects. So far, most data on farm animals have been generated in sheep.

Using embryo transfer, it was shown that B12 vitamin and folate deficiency in embryo donor ewes and transfer of these embryos in control females induces excess weight and adiposity in sheep offspring, insulin resistance, increased blood pressure and altered response to immunological challenges, as well as differences in liver methylation (Sinclair *et al*., 2007). The transfer of embryos, collected from restricted or obese ewes maintained in the same nutritional plane or induced to loose or gain weight in the last month before mating and for the pre-implantation period, into control recipients, showed that maternal restriction in the periconceptional period, regardless of previous nutritional status, resulted in adrenocortical hypertrophy (Zhang *et al*., 2013b) together with changes in the renin-angiotensin system regulation within the adrenal (Zhang *et al*., 2013a). Periconceptional undernutrition was shown to induce an increment in the body weight and the oocyte population of the offspring, as well as an alteration of their locomotor activity (Abecia *et al*., 2014).

Like for general nutrition, the effects of maternal undernutrition around conception vary depending on the number of implanted embryos. First, maternal weight loss as a result of periconceptional undernutrition in sheep has been shown to reduce twinning rate (MacLaughlin *et al*., 2005; Debus *et al*., 2012; Abecia *et al*., 2014). In general, birth weight is not directly affected by periconceptional nutrition. Indeed, uterine blood flow is increased by 13% in ewes that were undernourished during the periconceptional period compared to controls (Rumball *et al*., 2008) with increased expression of growth factors (Zhu *et al*., 2007).

Effects of periconceptional (-60 days before to 7 days after conception) or pre-implantation (0-7 days after conception) nutrition were observed on fetal skeletal muscle insulin signaling, lipogenesis, adipose tissue and liver metabolism as well as miRNA expression, depending on twin or singletons pregnancy (Lie *et al*., 2012, 2013, 2014). The timing and importance of fetal pre-partum ACTH and cortisol increases as well as gestational length were shown to differ depending on embryo number, with reduced gestation duration only in singletons (Edwards *et al*., 2002, 2004; Edwards and McMillen, 2002; Bloomfield *et al*., 2003). These effects may be related to the increased weight and disturbed function in the adrenal

observed in singletons in these and other studies (Connor *et al*., 2009; Williams-Wyss *et al*., 2014). Responses to glucocorticoids are also affected: hypothalamic glucocorticoid receptor promoter methylation, as well as gene and protein expression, were still observed in 5 year old male and female offspring, which could explain the increased obesity observed in these animals (Begum *et al*., 2013). Regardless of singleton or twin status, the cardiovascular function was impaired in adult sheep (Gardner *et al*., 2004; Torrens *et al.,* 2009).

Twins are generally smaller than singletons. Using an elegant approach of fetal reduction during pregnancy, Hancock *et al*. (2012) have shown that twins had lower lean mass and higher fat mass until 2 years of age compared to their contemporary singletons. Twin reduction to singleton pregnancy at 42-43 days of gestation did not recover the programmed twin fat and lean mass phenotype, indicating the early origin of these traits (Hancock *et al*., 2012).

As mentioned earlier, the genetic background of the animals induces large differences in the response to undernutrition. For example, gestational length was increased by periconceptional undernutrition only in twin-bearing Welsh mountain ewes carrying fetuses of opposite sex (Cleal *et al*., 2007). In another study, maternal periconceptional undernutrition did not reduce birth weight nor gestational length in the hardy Mediterranean breed Merinos d'Arles (Debus *et al*., 2012). Nevertheless, increased post-natal adiposity was observed in males but not in females, underlining the importance of offspring sex as well as litter size and breed (Debus *et al*., 2012).

In terms of response to post-natal nutritional treatment, it is interesting to observe that maternal nutrition at the time of conception was shown to directly affect lamb responses to nutritional supplementation with n-3 polyunsaturated fatty acid (PUFA)-enriched diets: offspring of dams fed a high n-6 fatty acid-riched diet for 6 weeks before mating had lower responses to algae supplementation (n-3 PUFA-enriched diet) compared to those born to control dams (Clayton *et al*., 2014).

In terms of behavior, maternal restriction from 60 days before to 30 days after the beginning of pregnancy was reported to be associated with decreased locomotion in 18 month old offspring (Donovan *et al*., 2013) whereas locomotion and attempts to escape were decreased during isolation after maternal periconceptional undernutrition, possibly reflecting decreased responses to stress (Hernandez *et al*., 2010; Abecia *et al*., 2014).

Conclusions

The pre- and periconceptional periods are critical in the context of the Developmental Origins of Health and Disease (DOHaD). Maternal *in vivo* environment, in particular nutrition, can disturb the apposition of epigenetic marks throughout gametogenesis, fertilization and the first steps of embryonic development, which are times during which major epigenetic changes take place (Jammes *et al*., 2011). These marks will subsequently affect organ function during development, resulting in alterations in the post-natal phenotype (Watkins *et al*., 2008; Watkins *et al*., 2010). The *in vitro* environment, in the case of assisted reproduction techniques, also affects epigenetic marks. Whilst the embryo is a target of these changes, female and male gametes are both target and vector of these epigenetic changes, thus leading to multigenerational effects, so that long-term consequences on the phenotype of offspring vary according to the sex of the vector parent, the sex of the individual and the generation (Aiken and Ozanne, 2014).

More work is needed to understand how the environment modulates the genomic inheritance in order to induce a phenotype and how this may be used in agriculture to lead to more robust animals able to tackle the climatic challenges that we will be facing in the future.

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Embryo metabolism: what does it really mean?

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Abstract

The study of early embryo metabolism has fascinated researchers in the field for nearly a century. Herein, we give a brief account of the general features of embryo metabolism and some consideration of the research performed to reach such conclusions. It is becoming increasingly obvious that metabolism informs many fate decisions and outcomes beyond ATP generation, such as DNA methylation, Reactive Oxygen Species generation and cell signaling. We discuss the reasons for studying metabolism in the face of our current knowledge of the effect that the culture environment on the developing embryo and the downstream effects that can cause. The study of *in vitro* embryo metabolism can also give us insight into developmental perturbations *in vivo*. The strengths and limitations of the methods we use to study metabolism are reviewed with reference to species-specific fundamental biology and plasticity and we discuss what the future holds for metabolic studies and the unanswered questions that remain.

Keywords: ATP generation, method evaluation, preimplantation development.

Introduction

The study of mammalian early embryo metabolism has a rich history (Leese, 2012). Whilst work in the period of the 1940s-1960s focused on the effect of adding energy substrates to embryos in culture, real progress in understanding embryo metabolism was made in the 1970s by the likes of Biggers and Stern (1973), Brinster (1973) and Gwatkin and Haidri (1974) who examined the fate of radiolabeled compounds added to the medium. From experiments such as these, a picture of early embryo metabolism began to emerge. Like so much of our knowledge of early mammalian embryo development, the first data came from the classical laboratory model species; mouse and rabbit, as well as the hamster. Interest grew, and embryo metabolism was soon examined in the large domestic animals; pigs, cattle, sheep and, to a lesser extent, the horse, dog and cat. Underpinning research were studies on early human development with the aim of clinical translation for the treatment of infertility; a feat first achieved in 1978 by Steptoe and Edwards. Alongside this feat was the development of assisted conception techniques for use in farm animals. It is not the intention of this article to re-describe the history of the research that lead to successful embryo culture or the contribution that studies on metabolism made. For expert insight, the reader is encouraged to read (Leese, 2012; Chronopoulou and Harper, 2014).

Embryo metabolism: what do we know?

The description of carbohydrate metabolism during preimplantation development is largely accepted and will be familiar to anyone who has an interest in the early embryo. In almost all species studied, the cleavage stage embryo, from fertilisation through to formation of the morula, is relatively metabolically quiescent. Oxygen consumption at this time remains comparatively low, and the dominant substrate depleted from the culture environment is pyruvate. Pyruvate is consumed at an almost steady rate during cleavage, with a proportion of the carbon (depending on the species) appearing in the medium as lactate with the generation of metabolic energy. The source of the pyruvate involved in such reactions is generally either glycolytic conversion of glucose or that taken up directly from the external environment. Pyruvate may also enter the Tricarboxylic Acid (TCA; Krebs) cycle, where it can be oxidised completely generating electron donors for the electron transport chain which occurs in the matrix of mitochondria and relies on oxygen acting as the terminal electron acceptor. For this reason, oxygen consumption provides a good marker of overall oxidative metabolic activity (for review, see Smith and Sturmey, 2013).

As the cleavage stage embryo progresses to a blastocyst, there is a sharp and characteristic rise in the amount of glucose consumed in all species studied, and a concomitant rise in lactate release into the medium. Coincident with this is a fall in pyruvate consumption. This general pattern of “blastocyst glycolysis” appears to be conserved across all species studied. There are a range of explanations for this, however, as glycolysis is a comparatively inefficient means of generating ATP therefore energy production is unlikely to be the prime reason. Moreover, as the blastocyst forms, oxygen consumption also rises (Fridhandler *et al.,* 1957; Houghton *et al.,* 1996; Thompson *et al.,* 1996; Trimarchi *et al.*, 2000; Sturmey and Leese, 2003)

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further supporting the notion that glycolytic production of lactate is of minor consequence in contributing ATP for the blastocyst. It is much more likely that glycolysis rises to meet the need for carbon for biosynthetic processes. A description of glycolysis in the early embryo can be found in Smith and Sturmey (2013). This general picture of embryo metabolism was summarized with great prescience by Brinster in 1973; in the intervening years many laboratories across the world have generated evidence to support such a description, illustrating the robustness with which these findings can be considered.

While early work focused on carbohydrate metabolism, it is now clear that the metabolism of amino acids, lipids and vitamins such as folate all also act in an interdependent manner to produce a viable embryo. Amino acids are crucial components of the culture environment *in vitro* (reviewed by Sturmey *et al.,* 2010). Their addition to simple culture medium either singly (Rieger *et al.,* 1992a) or in combinations (Chatot *et al.,* 1989; Gardner and Lane, 1993) permitted mouse embryos to be cultured past the so-called 2-cell block (Chatot *et al.,* 1989) and their widespread inclusion lead to improved blastocyst rates in almost every species studied. The addition of amino acids has had such a positive effect on the efficacy of *in vitro* embryo culture, that their inclusion is often described as having a primary role in the formulation of "next generation medium" (Leese, 2012). The precise mechanism for the positive effect of amino acid provision is still to be defined, however it is well established that addition of amino acids to *in vitro* medium can alleviate culture associated stress in flushed murine embryos (Lane and Gardner, 1998). The contribution that amino acid metabolism makes to ATP production remains unclear, however the turnover of amino acids (that is, the sum of their depletion or accumulation into the culture droplet) has been linked to embryo blastocyst rates (Houghton *et al*., 2002), human embryo live birth rates (Brison *et al.,* 2004), DNA damage (Sturmey, 2009), aneuploidy (Picton *et al.,* 2010) embryo sex (Sturmey *et al.,* 2009a), maternal age (Picton *et al.,* 2010) and embryonic stress (Wale and Gardner, 2012).

When considering energy metabolism of early embryos, it is vital that the contribution made by endogenous triglyceride is not overlooked. Fatty acid β-oxidation was studied in detail in the 1970s by Kane and colleagues (Kane, 1979) but then largely ignored, with the notable exception of the work by Downs (see Downs, 2015). However, interest in fatty acid metabolism has re-awakened, partly in response to the report from Dunning *et al.* (2010) who elegantly demonstrated that mouse oocytes require fatty acid oxidation in order to develop. A similar conclusion was drawn by Sturmey and Leese (2003) in the pig, underlining the importance of fatty acid β-oxidation during oocyte maturation, development and in the preimplantation stages. Species differences in the importance of fatty acid oxidation during oocyte and embryo development have also been identified. For example, where a mouse zygote will arrest after 15 h in media lacking nutrients (cited in Leese, 2012) a rabbit embryo can complete up to 3 cleavage divisions in the absence of energy substrates (Kane, 1987) and sheep embryos can also develop to the blastocyst stage in the absence of glucose (Thompson *et al.,* 1992). This can be explained by the differences in intracellular triglyceride content, acting in a buffering capacity by providing an alternate energy source (Ferguson and Leese, 2006; Sturmey *et al.,* 2009b). Recently, a number of laboratories have described altered fatty acid metabolism by embryos from overweight and obese mice (Pantasri *et al.,* 2015; Reynolds *et al.,* 2015) and the human (Leary *et al.,* 2014). After receiving comparatively little attention since the work of Kane, interest in fatty acid metabolism by oocytes and embryos has been intense, and has been widely reviewed in recent years (Sturmey *et al.,* 2009b; Leroy *et al.,* 2012; McKeegan and Sturmey, 2011; Dunning *et al.,* 2014; Downs, 2015).

This very brief overview is intended to remind the reader of the basic features of early embryo energy metabolism. However, 'metabolism' refers to significantly more functions than ATP generation. For example, there is an extensive literature describing the role of the pentose phosphate pathway (Downs *et al.,* 1998; Sutton-McDowall *et al.,* 2010) in mammalian oocytes and early embryos. Moreover, metabolic processes link to signaling mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species (Agarwal *et al.,* 2005) and gene expression in terms of establishment of epigenetic marks such as methylation and acetylation and post-translational modifications of proteins (DeBerardinis and Thompson, 2012). For example, defects in folate metabolism have been linked to methylation and epigenetic modifications affecting developmental competence (Xu and Sinclair, 2015). However, reviewing all of the literature on embryo metabolism in its broadest sense would require several articles and so in the remainder of this article, we will consider some more fundamental aspects.

Why do we study embryo metabolism?

Understanding the basic physiology and metabolism of the early embryo is a noble quest in itself that has fascinated researchers over the past decades. However, a major gap in our knowledge is the metabolism of the *in vivo* produced embryo, as well as the embryo *in situ*, which remain an elusive goal. We aim to gain information that can, and has been, translated into clinical practice in many ways; to design appropriate species specific culture media with the aim of producing viable healthy offspring; to design non-invasive methods for embryo selection for transfer and

shed light on metabolic perturbations occurring *in vivo*. Moreover, as our understanding of somatic cell nuclear transfer (SCNT; Wilmut *et al.,* 2002) grows and becomes linked inextricably to stem cell physiology and regenerative medicine, we must also accept that we know comparatively little about the impact of such techniques may have on embryo physiology. Furthermore, we are on the brink of many new and exciting developments in Assisted Conception, including mitochondrial transfer for the treatment of debilitating hereditary conditions as well as the replenishment of mitochondria in aged oocytes with the aim of improving pregnancy rates in older women (Craven *et al.,* 2010; Smeets, 2013). Such techniques may be considered 'beyond experimental'; mitochondrial transfer was licensed for treatment in the UK in 2014 and autologous mitochondrial transfer for infertility is already commercially available in some countries. However, since each of the approaches described above involve, in some way, altering the mitochondrial content of embryos, the need for detailed understanding of metabolic regulation of individual preimplantation mammalian embryo has never been greater.

A further drive to study embryo metabolism comes from the need to identify biomarkers of embryo health and viability. This relies on the inherent variability in metabolism between different embryos and has been used in an attempt to select viable embryos for transfer, with the end goal being clinical IVF in humans. There have been several observations that have yielded promising results. The 'quiet embryo hypothesis' proposed by Leese in 2002, stated that those embryos that are viable have a decreased metabolic rate; a proposition that has been supported by several studies showing embryos with an upregulated metabolism of both carbohydrates and amino acids to have decreased viability post transfer (Lane and Gardner, 1996; Sturmey *et al.,* 2009a; Guerif *et al.,* 2013). However, the notion is contested, and there are recent studies suggesting that elevated metabolism, particularly with respect to glucose consumption is associated with embryo viability (Gardner *et al.,* 2011). Clearly, this is an area in which more work is needed.

Since pioneering observations linking human birth weight to cardiovascular events in later life by David Barker *et al*. (1989) it has now been shown unequivocally in many species that the periconceptual environment can have downstream effects which can impact on the viability of the developing embryo and on the future health of the resulting offspring (Ceelen *et al.,* 2008; Watkins *et al.,* 2008; Leroy *et al.,* 2009; Fleming *et al.,* 2012; Frank *et al.,* 2014). It is also clear that certain embryonic stages are more susceptible to damage (Rieger *et al.*, 1992a), such as the early cleavage embryo during embryonic genome activation, suggesting that progeny may have a 'memory' of their origins.

With the rising obesity epidemic both in humans and companion animals, in addition to metabolic disease in farm animal species due to increased production pressures, the study of embryo metabolism *in vitro* can provide insight into the mechanisms of resultant suppressed fertility and potentially identify therapeutic interventions.

These are important reasons for studying embryo metabolism, and it is clear that metabolic processes can directly influence gene expression (Van Hoeck *et al.,* 2011, 2013), and patterning of the embryo (Leary *et al.,* 2014). However, it is also of fundamental importance to be aware of what is measured when studying embryo metabolism. In the final part of this review, we will describe the strengths and limitations of embryo metabolic studies.

What are we actually measuring?

The measurement of embryo metabolism is faced with many technical challenges. Critically, the *in vivo* environment is still largely unknown for most species, meaning that the extrapolation of knowledge to an embryo *in vivo* is of questionable validity. The data available on embryo metabolism inform us of the strategy of substrate depletion and appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited number of substrates at static levels; supply and ratio of substrates varies only in response to an embryo's own activity. This is in stark contrast to the situation *in vivo*, which is dynamic and responsive (Leese *et al.,* 2008). Even in species for which the *in vivo* embryo environment has been described, the method used to define it should be noted. Often *post mortem* changes and/or inflammatory changes due to catheterization can influence results thus making samples non-representative (Leese *et al.,* 2008). Moreover, the embryo *in situ* likely exists in a microenvironment within the oviduct, thus any subtle, specific composition features will be lost in flushing of the tube.

Given the heterogeneity in developmental potential, measures pertaining to single embryos are key and thus highly sensitive assays are needed. Both the use of radiolabelled substrates (Rieger *et al.,* 1992b) and enzyme-linked fluorescence assays to detect the appearance and disappearance of a substrate from culture media have been described (Leese and Barton, 1984; Guerif *et al.,* 2013). The relative metabolic quiescence of single embryos means that 'analysis media' (that is a medium in which the concentrations of substrates is reduced to enable measurement of change) is often used in order to permit detection of changes in substrate concentration (Hardy *et al.,* 1989; Sturmey and Leese, 2003). This 'analysis medium' is often different to the *in vitro* culture media known to support development for most species, which, in turn differs vastly to the *in vivo* environment. Of course, it also must

be realized that there are many complex cell transport and metabolic pathways involved, and notions of influx and efflux leads' us to make what are essentially educated guesses about what occurs in the cell. Despite these limitations, these assays have greatly advanced our knowledge of metabolic pathways involved and have yielded highly repeatable results across different laboratories. Further methods that have been used to detect metabolic activity of embryos include culturing individually in micro-droplets or in large groups of embryos. However, the resolution of data from group culture is reduced since individual embryo heterogeneity is lost by 'averaging'.

New promising studies using NMR metabolomic technology, where substrate flux can be measured *in situ* have been recently described (Krisher *et al.,* 2015), however the subsequent interpretation and analysis of the complex data acquired presents new challenges.

Inferences about the contribution of oxidative metabolism are usually derived from measuring oxygen consumption. Methods vary, the most widely used being pyrene fluorescence (Houghton *et al.,* 1996) and nanorespirometry (Lopes *et al.,* 2010). Again while allowing accurate measurement of oxygen depletion in single embryos and seemingly not affecting development (Lopes *et al.,* 2005), the methods represents a significant 'alien' environment for the embryo.

Studies involving metabolic inhibitors and enzymatic co-factors have also added to our knowledge of embryo metabolism and in some cases provided the initial proof of certain pathways occurring and either being essential or non-essential for development. Among these, Brison and Leese (1994) showed that oxidative phosphorylation was not an absolute requirement for blastocoele formation in the rat by culturing embryos in the presence of cyanide, while Macháty *et al*. (2001) indicated that suppression of oxidative phosphorylation at the morula stage improved development to the blastocyst in the pig. Moreover, Dunning *et al.* (2010) have shown that β-oxidation is essential for optimal development in the mouse by culturing in the presence of etomoxir. In some cases, inhibition of certain metabolic pathways has been shown to improve developmental potential; for example the addition of EDTA to embryo culture medium (Gardner *et al.,* 2000). Although the mechanism is not confirmed, one possible role of EDTA in embryo culture medium is the suppression of glycolysis (Gardner *et al.,* 2000). However, it is equally likely that EDTA acts as an antioxidant by sequesteration of metal ions which would otherwise catalyse the formation of Reactive Oxygen Species (Orsi and Leese, 2001). Studies such as these illustrate the importance of appropriate regulation of metabolic pathways during development and also indicate why it is necessary for pathways to be correctly orchestrated to match needs at a given stage of development.

It all depends on the environment

It could be argued that measuring embryo metabolism *in vitro* (by necessity) amounts to measuring a stress response. This issue must be considered given the extremely adaptable nature of embryos of all species. Metabolism is necessarily dynamic, enabling rapid changes in needs to be met to maintain development. However, such dynamism means that the metabolic profile of an embryo can respond quickly in response to a change in external environment, shown clearly in mice, where perturbations occur within 3 h of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the presence and relative quantities of metabolic substrates in the environment in which experiments are conducted will significantly affect the results. While not attempting to provide a detailed discussion on the controversial aspects of *in vitro* culture systems, which still vary widely across laboratories, this point can be further illustrated by the differential metabolism that results from the presence or absence of serum and the atmospheric oxygen concentration (Wale and Gardner, 2010).

While the human IVF industry has moved towards defined culture media using macromolecular sources such as recombinant albumin, serum is still used in many production animal systems. Culture with serum has been shown to increase blastocyst development rates in the horse (Choi *et al.,* 2004) and the kinetics of blastocyst development in the cow (Rizos *et al.,* 2003). However, its presence has also been associated with increased intracellular lipid content (Ferguson and Leese, 2006) and altered metabolism (Reis *et al.,* 2003), up-regulation of oxidative stress and inflammatory pathways (Cagnone and Sirard, 2014) and decreased survival after vitrification (Gómez *et al.,* 2008). In addition, the oxygen tension of the reproductive tract in all species studied has been found to be below 10% (Fischer and Bavister, 1993). In terms of the environmental gas profile, there is now unequivocal evidence to support the notion that 20% oxygen reduces embryo development (Thompson *et al.,* 1990; Wale and Gardner, 2010) and that culture in low oxygen (5%) results in metabolic and proteomic profiles more closely matching *in vivo* counterparts (Thompson *et al.,* 1990; Katz-Jaffe *et al.,* 2005). Clearly, these factors will influence the results of any metabolic study and must be kept in mind when comparing studies.

In addition to the embryo adapting to its environment, the culture environment itself is not static. Depletion and accumulation of excreted of substrates such as lactate and amino acids will change the local environment. Spontaneous de-amination will occur at 37°C, especially of glutamine, resulting in ammonium build up (Gardner and Lane, 1993), lactate build up may

overwhelm pH buffering system of the media and depletion of energy substrates can lead to alternative ATP generating pathways being used (Kane, 1987).

It is also important to note that the manner in which an embryo responds to its environment is species specific. This can be seen in differences in response to hyperglycaemia. While species such as rodents and humans, will have significant diminished development in the presence of high glucose (Moley *et al.,* 1998; Frank *et al.,* 2014), others such as the horse and pig are apparently unaffected (Sturmey and Leese, 2003; Choi *et al.,* 2015). Qualitative testing of equine embryos produced in hyperglycaemic conditions however, highlights subtle differences not reflected in the blastocyst development rate such as a decrease in ICM cell number allocation (also observed in the rat) and known to be mediated through apoptosis (Moley *et al.,* 1998; Choi *et al.,* 2015).

It is thus vital to consider that studies on embryo metabolism provide us a snapshot of physiology in a given set of conditions. Whilst such data are of fundamental importance, care must be taken when extrapolating and comparing such information. It is thus much more desirable that studies on the depletion and appearance of embryo metabolism are reinforced by consideration of mechanisms of metabolic regulation of early development.

Embryo metabolism: some unanswered questions

As the emphasis in human IVF is increasingly on single embryo transfer, the identification of reliable non-invasive methods of determining embryo quality to maximize pregnancy rate per transfer remains the Holy Grail. Moreover, in species such as the horse where *in vitro* embryo production is rapidly generating interest, a specific tailored culture media has yet to be formulated. Whilst acceptable blastocyst rates (41%) and pregnancy rates after transfer (66%) can be achieved by some laboratories in the horse using cell culture media such as DMEM-F12, (Jacobson *et al.,* 2010; Hinrichs *et al.,* 2014) the more subtle effects of potentially inappropriate culture conditions leading to decreased viability remain to be seen. Identifying optimal species-specific culture systems presents an exciting challenge for those involved in studying embryo metabolism.

Sex selection is another lively area of embryo metabolism. Ethical considerations preclude the implementation of sex selection in the human, but in the production animal industry, and in dairy cattle in particular, appropriate non-invasive identification of sex before transfer would be an application with many uses. Promising results have been presented so far showing that both glucose metabolism and amino acid metabolism varies with sex (Sturmey *et al.,* 2010; for review see Gardner *et al.,* 2010), however more work will need performed to increase specificity in order for the technology to make the transition to commercial practice.

New information is emerging all the time on the far-reaching downstream effects of aberrations in early embryo metabolism (Harrison and Langley-Evans, 2009). Given the clear links between the periconceptual environment and sub-optimal health outcomes in the human (Barker *et al.,* 2002) and production species such as the bovine (for example, the so-called Large Offspring Syndrome; Young *et al.,* 1998), understanding and attempting to mitigate the negative effects on suboptimal embryo development and life-long health of the offspring is an important area for future study (Leese, 2014).

Conclusions

It is acknowledged"that metabolism pervades every aspect of cell physiology" (DeBerardinis and Thompson, 2012) and this is especially pertinent to the developmentally plastic early mammalian embryo. As genomic, transcriptomic and imaging techniques advance we will be able to expand our understanding of embryo metabolism and how it links inextricably with developmental pathways through subsequent stages of gestation leading to the birth of a healthy offspring. It is the responsibility of us all working in the earliest stages of this process to understand the periconceptual environmental challenges faced by the embryo and to optimize the conditions under which it is grown to ensure the best start in life. Metabolic studies allow us to gain vital information on the requirements of a competent embryo and identify when things go wrong, but the reader is cautioned towards careful interpretation of measures of metabolism especially between laboratories and to consider the environment as a whole under which they have been taken.

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Molecular strategies for gene modification in livestock

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Keywords: genetic engineering, genome editing, transgenesis.

The use of reproductive biotechnologies in livestock over the past 50 years has had a productive impact of enormous magnitude, with great consequences on food production in terms of quality and quantity. One of the biotechnologies developed in the last two decades, transgenesis, is positioned as a set of technologies applied to animal assisted reproduction. This technique emerges as a tool that goes beyond animal production, as it not only includes these aspects, but also incorporates animals as source of food with special qualities (nutraceuticals) and/or of drugs for animal and human health (pharmaceuticals). Biopharmaceutical production is useful for a variety of purposes, including the expression of enzymes important in human nutrition. Until 2012 transgenic domestic animals were produced mainly by inserting a vector containing the gene or genes of interest in cultured donor cells for somatic cell nuclear transfer (cloning). Currently, new genetic tools are being developed and allow the manipulation of the genome, making more efficient mutation events directed by a cut in the double-stranded DNA. As a result, the cell recruit endogenous repair machinery, and this can be repaired by homologous recombination or non-homologous end joining, thereby generating a reliable arrangement, a deletion or insertion of nucleotides. The last two options produce a change in the open reading frame generating premature stop codons in two thirds of cases. Normally this type of transcript is degraded by a mechanism known as Nonsense Mediated Decay (NMD). One such tool, CRISPRs-Cas9 comes from bacteria and once inside the plant cell are capable of activating genes, thus creating better conditions for their survival and replication. It has been shown that artificially designed CRISPRs are able to recognize and bind to specific sequences and are capable of activating the transcription of specific genes, thus opening the door to a wide variety of applications in genome engineering.

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Workshop: III Symposium of the South American Research Consortium on Cloning and Transgenesis in Ruminants

Transgenic animals for the production of therapeutic proteins in the milk

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Keywords: bioreactors, genetic engineering, livestock.

Therapeutic proteins have a long history, initially being extracted from animals or even from human sources. With the approval of recombinant human insulin in 1982 by FDA, the first approved therapeutic recombinant protein, a huge market for recombinant therapeutic proteins was envisioned. Since then, more then 200 recombinant drugs have been approved in the USA and European Union, representing an increasingly important group of pharmaceuticals. The term biopharmaceutics started to be used in the 1980s to define products generated by recombinant DNA technology or genetic engineering, which has dominated the biotech industry in the last 20 years. More specifically, the recombinant production of therapeutic proteins for the treatment of complex human diseases is a major force linked to transgenic animal production. This class of drugs is currently the largest source of innovation in the pharmaceutical industry. Over the past decade, the market for biopharmaceuticals maintained a steady growth of around 13.3% per year accompanied by a 4.2% decrease in the same period for other types of drugs. In addition, biopharmaceuticals account for 55% of all new approved drugs and 64% of drugs in development and/or approval. The marketing growth became the driving element of all efforts related to the development of new therapeutic proteins of biological origin, in which, the transgenesiscan constitute a key component. Among the systems used in the search for production of biopharmaceuticals, the development of animal platforms based on the use of transgenic animals offers particularly attractive possibilities. The main advantages lie in low production costs combined with high productivity and the quality of the synthesized proteins. Currently, the recombinant protein expression in milk is the most robust system for the production of biopharmaceuticals from transgenic animals. Within this scope, transgenic goats have been widely used due to several advantages. The expression system in the mammary gland is the only one within the animal platform to generate recombinant proteins as legalized trade for therapeutic use, asAtryn® (rREVO Biologics Inc), a recombinant antithrombin produced in the milk of goats, was the first biopharmaceutical produced from a transgenic animal approved for human treatment in 2006 by the EMA (European Medicines Agency) and in 2009 in the US-FDA (Food and Drug Administration). More recently, rabbits have joined the goats in the approval of another biopharmaceutical from milk, Ruconest® (Pharming), a recombinant human C1 esterase inhibitor protein (C1INH). Following a similar path and trend, Ruconest was first approved by EMA in 2012, followed by the FDA approval in 2014. This revolutionized the landscape of scientific and marketing biopharmaceutical possibilities and added much to the reliability of recombinant protein production system through animal platform using the expression system in milk. We have been engaged in recent years in the establishment and development of in vitro and in vivo studies using transgenic animal models (goats, cattle) to produce animal milk containing therapeutic proteins to be used as biosimilar drugs and also to produced recombinant vaccines for use in humans and animals. The mammary gland transgene expression systems for the production of functional proteins in the milk of animals have been proven as a viable technological alternative to aid in the resolution of problems of the modern world.

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Conceptus metabolism and systemic physiology in cloned cattle

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Keywords: Abnormal Offspring Syndrome, *in vitro* fertilization, somatic cell nuclear transfer.

Improvement of fertility in agriculturally-important species will have a direct impact on key segments of the livestock industry and agribusiness around the world, as well as on the environment and animal well being. One of the most critical aspects of infertility in production animals is the failure of a female to deliver viable offspring. That scenario is a typical feature of intensive production systems, as in dairy cattle, for which such failures may exceed 60% from ovulation to term. Lower pregnancy rates with higher gestational losses result in lower prolificacy, and represent a huge economic loss. This is an example of where basic and applied science should converge to the benefit of knowledge and the livestock industry. *In vitro* embryo production (IVP) in livestock species by *in vitro* fertilization (IVF) or cloning by nuclear transfer (NT) procedures have been of extraordinary importance for research and development, providing new knowledge, insights, and strategies for the resolution of fertility problems. In such scenario, Brazil has been leading the commercial IVF activity in the world, by the transfer of more than 300 thousand IVF embryos a year. However, the IVF and NT technologies are often associated with increased rates of pregnancy losses, developmental abnormalities, and birth of large and abnormal offspring with lower postnatal survival. As the Abnormal Offspring Syndrome (AOS) limits the practical use of some *in vitro* technologies, and as the physiological basis for the abnormalities is still widely unknown, IVF and cloning procedures provide excellent tools for the study of many biological processes that need to be elucidated in order for abnormalities following *in vitro* embryo manipulations can be fully understood. Recent changes in IVP systems for IVF procedures, especially for *in vitro* embryo culture conditions, have been minimizing problems, with conceptus traits in IVF-derived concepti and newborns getting to be similar to controls, with slight changes in placentome development that does not seem to affect conceptus growth and survival. However, cloning by NT continues to unpredictably result in abnormal phenotypes and higher morbidity and mortality after birth. In a systematic study on the AOS in cattle, we have shown significant differences in conceptus development between *in vivo*-derived and IVP pregnancies, including fewer but larger placentomes and increased placental surface area during late pregnancy, increased glucose and fructose accumulation in fetal fluids, with a seemingly effect on life *ex utero*. Pregnancy outcome may be similar to controls, but pregnancy losses in the first trimester can be greater than 70%. A biphasic growth pattern is commonly seen, with early conceptus being initially smaller than controls, but significantly larger and heavier in late pregnancy and after birth. Usually, a higher frequency of enlarged placentomes (more than 60%) may be observed after cloning, in an apparent greater role of less frequent and abnormal, nonetheless heavier, placentome types in placental function. We have associated the occurrence of enlarged IVP concepti with changes in metabolic and molecular profiles in the fetus and/or placenta in late pregnancy and after birth. Significant difference exists in activity in metabolic pathways and placental function in cloned concepti, suggesting an active glucose synthesis, an increase in fructose synthesis by the placenta, and in fructose catabolism by the fetus, which may be a reflection of an association between changes in metabolic fetal programming and excessive prenatal growth after cloning. In addition, *in vitro*-derived newborn calves may be heavier and larger at birth, having lower respiratory rate and thermoregulatory response than *in vivo*-derived controls, with higher concentrations of fructose in the immediate period after birth. In general, physiological and metabolic findings at birth may be widely similar and normal when compared with controls. However, during the first 24 h of life, IVF-derived calves showed physiological, metabolic, biochemical, hemogasometric, and hematologic features indicative of a lower adaptation to life *ex utero*, particularly in the first 4 to 6 h of life. The main differences indicate that larger animals, mainly IVP-derived calves, have more difficulties to maintain plasma oxygen levels, with evidence of a metabolic shift and elevation of metabolic substrates (mostly lactate), and a trend for acidosis followed by a compensatory normalization of the acid-base balance, predominantly in the first 6 h of life. Also, the fructose metabolic pathway was shown to be active in *in vivo*- and *in vitro*-produced newborn calves in the first hours of life. Consequently, the presence of pregnancy-derived higher fructose levels in the plasma of newborn calves may be metabolically and clinically beneficial for the postnatal adaptation of the life *ex utero* under uneventful conditions, but deviations from physiological normality, such as respiratory distress, may cause profound metabolic consequences that might compromise postnatal survival, leading to distress and death, an event more commonly seen in *in vitro*-derived newborn calves. In summary, morphological changes in cloned concepti likely influence placental function and metabolism, disrupting the placental constraining mechanism on fetal growth, leading to accelerated conceptus growth detectable in late pregnancy. Problems associated with cloning and IVF have already propelled our curiosity for the understanding of causal factors, which in turn has contributed to numerous advances and generated great knowledge in many fields, such as the physiology of pregnancy, placental function, epigenetic reprogramming, transgenesis, neonatology, etc. Such research will continue to increase our understanding of developmental processes of physiological (e.g., placentation) or pathological (e.g., embryonic mortality) interest. Thus, new knowledge may become applicable in the future, with direct economical implications for livestock production, by providing new insights for ways to minimize losses and increase fertility in agriculturally-important species.

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Production of recombinant vaccines in the milk for human and animal diseases

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Keywords: genetic engineering, transgenesis, vaccines.

Zoonotic diseases can be passed between animals and humans and are caused by a variety of organisms as viruses, bacteria, parasites and fungi. The spread of zoonotic diseases occurs at the human animal interface, which in most cases is the close contact between livestock and farm workers. In developing countries, the lack of proper sanitation and processing of food products increases the likelihood of disease transmission between livestock and humans. It is estimated that more than 6 out of 10 infectious diseases in humans are spread from animals. Moreover, increasing emergence of drug resistant strains of bacteria, stemming from improper use of antibiotics in human and veterinary medicine, as well as livestock production, escalate the risk to both humans and animals. The most effective means of eradicating zoonotic diseases would be to develop vaccination strategies that could be implemented simultaneously in both the human and animal populations, which is the essence of the One Health philosophy. Multivalent subunit vaccines have been utilized in a disease specific manner. However, there has never been a published attempt to produce a single vaccination platform containing multiple antigens to multiple pathogens that can be used in both humans and livestock. In such scenario, the transgenic animal platform of expressing recombinant proteins in the milk offers particularly attractive possibilities. The main advantages lie in the triad that combines the low cost of production with high productivity and quality of the produced proteins. Probably this triple alliance brings together the best attributes related to the success of any biopharmaceutical production platform. Recently, we have launched the first steps in the direction for setting up a production platform for recombinant vaccines in the milk of animals that allows us to quickly produce and test different antigens and allocate the best immune response to large-scale production using mammary gland as a bioreactor, offering protection from multiple pathogens in a single vaccine. By applying a transient approach, subunit vaccines can be screened and developed to target multiple pathogens and can be effective for both humans and animals. The mammary gland can efficiently produce large quantities of affinity tagged antigens capable of efficient low cost purification and production into a single effective vaccine for humans and animals. Furthermore, and perhaps most importantly, once the animals are genetically modified produced, they can be propagated, milked and the vaccines processed in the areas of need, which can further reduce cost and provide economic stability in developing regions, offering protection from multiple pathogens in a single vaccine. We believe that designing gene expression constructs capable of producing dozens of affinity tagged peptides offers the best approach for the development of a single vaccine that can block transmission of many devastating zoonotic pathogens anywhere in the world.

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Human and porcine induced pluripotent stem cells as disease models

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Keywords: animal models, iPSC, stem cells.

Neurodegenerative disorders with Alzheimer's disease (AD) being the most prominent are worldwide increasing in frequency. We aim at establishing *in vitro* cell models as well as whole animal models for AD. We have refined a reprogramming strategy that allows for human fibroblasts from patients with AD to be reprogrammed into integration-free induced pluripotent stem cells (iPSC). During iPSC reprogramming the fibroblasts undergo a very well orchestrated mesenchymal-to-epithelial transition. The patient-specific iPSCsare subsequently differentiated into neurons, which express certain phenotypic characteristics of AD in the petri dish. The diseased neurons can be utilized for drug development. Furthermore, molecular tools as e.g. CRISPRsnow allow for correction of mutations that cause the disease in familial cases. Hence, gene edited isogenic control cell lines can be established and strictly compared with the disease lines on the same genetic background. In the future, iPSC-based therapy for e.g. Parkinson's disease will be realized. A large animal model for studying the potentials and safety is needed. In order to pave the way for this, we have focused efforts on establishing porcine iPSCsas a model. We have tried different avenues and found that pluripotency transcription factors controlled by doxycycline-dependent promoters may drive temporary iPSC characteristics, which, unfortunately, fade when doxycycline is removed. However, using the plasmid-based reprogramming technology, applied to human reprogramming as above, we have been able to establish stable porcine cell lines with iPSC characteristics. Moreover, we found that a subfraction of porcine fibroblasts expressing the surface marker SSEA1 are particularly prone for iPSC reprogramming. In future projects we will also investigate canine cognitive dysfunction, a spontaneous condition in dogs with great similarities with AD, in order to develop the dog as a potential model for AD.

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Perspectives and cloning outcome at In Vitro-Brazil Clonagem Animal S.A.

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Keywords: equine, ICSI, somatic cell nuclear transfer.

Somatic cell nuclear transfer has been adapted from frogs for use in sheep as well as for use in many other mammalian species including bovine and equine. The birth of Brazils first 3 cloned foals resulted from work performed while developing equine oocyte maturation, cell synchronization techniques as well as embryo culture and embryo transfer medium optimized for use in equine based off our current bovine cloning method which uses donor cells synchronized in G2 of mitosis with oocytes in Telophase II of meiosis. This cloning approach differs significantly from that used in the creation of Dolly. Our approach adapting bovine cloning methods for use in the equine was initially focused only on embryo culture media, maturation components and time of activation. This proved insufficient, as embryo development was poor and not suited for a commercial enterprise. We moved forward the following year by further optimizing our equine cloning protocols from oocyte maturation medium to equine embryo transfer medium and all procedures between. In Vitro Clonagem provides four products: complete services for bovine and equine cloning, equine ICSI as well as cell line establishment and cryopreservation. Each of our cloning services guarantees timely delivery of the cloned bovine or equine based on a contractual agreement. Cell line establishment guarantees the animal genetics can be safely preserved well after the original animal dies. At the present time, equine ICSI is the one service where we can send a pregnant recipient back to the owner's farm after 90 days. Thus far we are observing more interest in equine cloning and especially in equine ICSI. As IVF in equine is not yet a viable option due to low fertilization rates, equine ICSI represents an alternative for continued reproduction in older mares. As for now, we can only provide services to replicate genetics brought to us from clients. In the future, we hope to offer services that help advance genetic improvement by offering gene editing and IVF trophectoderm biopsy services with our parent company, In Vitro Brasil. Visit us at http://invitrobrasil.com.br/en/cloning.php or email me directly at marc@invitrobrasil.com.br for more information.

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Phenotypic characteristics of F1 generation of transgenic goats producing hG-CSF in milk

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Keywords: goats, recombinant protein, transgenesis.

The discovery of DNA has opened many areas of research including biotechnology and genetic engineering. The possibilities of isolation and introduction of genes into cells has become a routine procedure in laboratories around the world. Recombinant proteins of high economic value have been expressed in genetically-modified mammalian cells, which are better prepared for the synthesis of complex proteins. However, the need of large capital investment, high operating costs and relatively low production levels result in the inability to produce more than a few kilograms of protein per year (Houdebine. J Soc Biol, 203:323-328, 2009). Given these limitations, the transgenic animal platform in which the recombinant protein is expressed usually in their mammary gland and thus purified from their milk, appeared as a promising method due to some features, such as low operating costs and virtually unlimited capacity to scale-up by simply breeding transgenic animals (Kues and Niemann. Prev Vet Med, 102:146-156, 2011). Human granulocyte-colony stimulating factor (hG-CSF) is a cytokine of high economic value currently produced in bacterial and Chinese hamster ovary (CHO) cells for clinical use. This cytokine is a glycoprotein that influences the proliferation, survival, maturation and functional activation of cells from the neutrophilic granulocyte lineage. Its main clinical application is to reduce the time of neutropenia (Creaet et al. Crit Rev Oncol Hematol, 72:21-44, 2009). Our group reported the production of two transgenic goats containing hG-CSF fused to goat α-S1 casein promoter (Freitas et al. Small Rum Res, 105:105-113, 2012). Later, we have demonstrated that the female founder successfully expressed the recombinant protein in her milk (Moura et al. Animiotechnol, 24:10-14, 2013). The aim of this study was: a) to verify the hG-CSF expression during lactation of F1 goats; b) to investigated the ectopic expression by ELISA and by qRT-PCR. It was used transgenic (n = 6) and non-transgenic (n = 6) goats that received a hormonal treatment for induction of lactation. Despite the lower milk production, transgenic females presented asimilar milk composition (fat, protein and lactose) when compared to non-transgenic ($P > 0.05$). The mean concentration (±SD) of recombinant hG-CSFin milk during lactation was 360 ± 178 μg/ml. All clinical parameters, as well as kidney and liver function, indicated that F1 transgenic goats were healthy. Additionally, no ectopic hG-CSF expression was detected. Thus, F1 hG-CSF-transgenic goats can express there combinant protein in milk at quantities compatible with their use as bioreactors in a commercial-scale protein-production program.

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Perspectives and cloning outcome at cenatte embriões/semex

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Keywords: calves, somatic cell nuclear transfer, zebu cattle.

Nuclear transfer is a biotechnology with emerging commercial applications and its efficiency has been improved despite the low productivity. The low rates of healthy clones are attributed to high incidences of placental abnormalities leading embryonic and fetal losses (Hossain et al. BMC Genomics, 43:1-15, 2014). Aiming to demonstrate the efficiency of CENATTE Embriões/SEMEX team, we monitored 946 cloned embryo transfers produced from 22 cell donor animals (20 female and 1 male) from October 2009 to August 2013 (breeds: Gir, Brahman, Guzerá, Nelore and Tabapuã). The fibroblasts were obtained by explants from caudal fold and the cloning technique was performed with micromanipulators according to Campbell et al. (Cloning and Stem Cells, 3:201-208, 2001). Efficiency rates were evaluated by pregnancy rate, gestational losses and production of healthy clones within 3 months after birth. From 946 embryo transfers, 42.3% (400/946) recipients were pregnant at 30 days and 22.3% (211/946) at 60 days post embryo-transfer. The gestational losses reached 47.2% (189/400) between 30 and 60 days and 78.5% (314/400) between 30 days to term. The birth rate was 8.9% (84/946) with 78 clones alive and six stillborn. The pregnancy and gestational losses data are similar to those presented by Heyman et al. (Biol Reprod, 66:6-13, 2002), which obtained 33.5% (45/133) of pregnancy at 35 days, 57.7% (26/45) gestational losses between 35 and 70 days and 80.0% (36/45) between 35 days and parturition. We found a postnatal survival rate of 73.1% (57/78). However, 30.8% (24/78) of calves had some form of physical defect. Considering the total of 946 ETs, we delivered to the farmers one male and 32 female healthy clones at third month after birth, representing an efficiency of 3.5% (33/946) for the entire process of commercial bovine cloning. This rate is similar to literature data that shows that less than 5% of embryos clones become healthy calves (Palmieri et al. Vet Pathol, 45:865-880, 2008). Considering the different breeds used by our company, the cloning efficiency was 4.6, 4.4, 1.2, 2.4 and 9.4% to Gir, Brahman, Guzerá, Nelore and Tabapuã, respectively. The cloning efficiency variation among cell donor animals was 0.87 to 12.5%. However, 31.8% (7/22) of the cell donors never produced healthy calves. To enhance the viability of clone calves is necessary to improve the techniques of nuclear transfer and neonatal care as well. This would allow a greater productivity gain of the entire commercial cloning technique in bovine.

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A001 Physiology of Reproduction in Male and Semen Technology

Morphological changes in sperm cells of stallions of the nordestine breed after thawing

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Keywords: equine, major defects, minor defects.

In spite of its importance for the Northeast region of Brazil, the Nordestino breed is reduced to a few animals, making studies on seminal characteristics, response to cryopreservation and post-thaw fertility of sperm necessary for breed preservation programs. This study evaluated the post-thaw sperm morphology in Northeast breed stallions to estimate fertility and feasibility for commercialization. This study was approved by Ethics Committee in humans and Experimental Animals (CEDEP) of UNIVASF (Protocol number 0006/161012). Were evaluated 19 ejaculates from two stallions (N = 10 and N = 9 ejaculates, respectively), clinically healthy, fertile and with an average of 9.5 years. Two days after the depletion of the extragonadal sperm reserves the ejaculates were collected, diluted (1:1) with Botu-Semen® (Biotech Botucatu, Botucatu, SP, Brazil), kept at 37°C and sent to the protected CPSENS protected from the light, where they were centrifuged in conical tubes (50 mL) at 2200 rpm for 15 min. The supernatants were discarded and the pellets resuspended in Botu-crio® (Biotech Botucatu, Botucatu, SP, Brazil) to the concentration of 106 sperm per mL and evaluated for total motility (TM) and progressive (PM) using CASA. Every ejaculate presented >70.0% of TM and was frozen by the traditional method and stored at -196ºC. After thawing in a water bath at 37°C for thirty seconds, a rate of 10 µL of semen was added to 1.0 mL formalin-buffered saline, heated to 37°C, and stored at room temperature for morphology analysis by the method of wet chamber. One drop of this mixture was placed between slide and coverslip and 200 cells were evaluated in 1000x magnification under phase contrast microscope. The total sperm defects were divided into major and minor defects (CBRA 2013). The average percentage of total defects was 5.86±4.22%, where the average of the defects was 2.12±1.93% of major and 3.74±2.29% of minor defects. The major defects were divided into: strongly coiled tail (4.14±2.31%), proximal cytoplasmic droplet (2.58±1.63%), head isolated (1.69±1.49%), incomplete acrosome (1.31±1.19%) and coiled tail (0.89±0.82%). The minor defects were simple coiled tail (5.14±1.93%), thin head (4.58±2.09%), elongated head (3.86±1.78%) and distal cytoplasmic droplets (1,39±1,34%). The results indicate that the analyzed ejaculates of these stallion of the Nordestine breed exhibits excellent morphological quality.

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A002 Physiology of Reproduction in Male and Semen Technology

Evaluation of integrity of acrosomal and plasmatic membranes and the mitochondrial potential of cryopreserved bovine sperm with or without the presence of seminal plasma

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Keywords: centrifugation, filtration, Nellore.

The post-cryopreservation sperm survival rates are not good for most species. In bovine, on average, around half of all the spermatozoids are damaged or destroyed by the freezing, limiting the total efficiency of the semen preservation. As a result, new strategies for cryopreservation are necessary to increase the number of living sperms and the quality of these ones after thawing them. One of the most used strategies for several species is the removal of seminal plasma before cryopreservation, which can be done through centrifugation or filtering of semen. Thus, the aim of this study is to evaluate the integrity of the acrosomal and plasmatic membrane and the mitochondrial potential of cryopreserved spermatozoids in 38 Nelore bulls with or without the presence of seminal plasma. After the semen was obtained through electroejaculation, it was fractioned in three equal aliquot and then the treatments were performed according to Campanholi et al. (VII Congresso Interinstitucional de Iniciação Científica (CIIC), Campinas, São Paulo, Brasil, 2013, p.1-8). Treatment T1 (traditional) consisted of the dilution of semen for the final concentration of $60x10^6$ spermatozoids/mL with dilutor BotuBov® (BotuPharma®, Botucatu, Brasil). Treatment T2 (centrifugated) involved a 10-minute centrifugation at 600xg (2200 rpm) to remove the seminal plasma. Treatment T3 (filtered) consisted of filtering the semen through Sperm Filter® (BotuPharma®, Botucatu, Brasil). After centrifugating and filtering the semen, the spermatozoids were resuspended with dilutor BotuBov® in the same concentration as T1. After all the treatments had been performed, the semen was envased at room temperature in pallets (0.5 mL) and frozen using TK 4000® (TK®, Uberaba, Brasil). The propidium iodide probe (Sigma®), FITC-PSA (Sigma®) and Hoescht 33342 (Sigma®) were used to assess the integrity of the acrosomal and plasmatic membrane whereas the probe JC-1 (Molecular Probes®) and H33342 were used to assess the mitochondrial potential. The analyses were performed through flow cytometry using BD® LSR II (Becton Dickinson, Mountain View, CA, USA) and the data were assessed through the program BD FACSDiva™ Software v6.1. The statistical analyses were performed using PROC GLM of SAS. A significance level of 5% of probability has been considered. T2 and T3 have shown a higher percentage of cells with damaged acrosomal and plasmatic membranes (T1=30.61±0.98, T2=38.46±0.98 and T3=39.34±0.98; $P<0.05$). There was no difference between treatments concerning the percentage of spermatozoa with high (T1=24.28±1.68, T2=28.22±1.68) and low (T1=33.32±2.06, T2=34.03±2.06 and T3=33.70±2.06; $P=0.97$) mitochondrial potential. Therefore, it has been concluded that the removal of seminal plasma has increased the percentage of damage in plasmatic and acrosomal membranes.

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A003 Physiology of Reproduction in Male and Semen Technology

Cooling systems evaluation in sperm kinetics and integrity of plasma and acrosomal membrane of bovine semen

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Keywords: CASA, Nellore, sperm quality.

One of the main steps in semen cryopreservation process is the cooling curve. The cooling rate may decrease the thermal shock and the damage to sperm cells, and the best results can be obtained using slower and homogeneous cooling curves. In this context, the objective was to compare simultaneously different bovine semen cooling systems and evaluate their effects on sperm quality. Thirty-eight ejaculates from 12 Nellore bulls (3.02±0.5 years of age, 631 ± 119 kg of body weight, and 33.8 ± 1.89 cm of scrotal circumference), collected by eletroejaculator, were analyzed. The samples were diluted in the means of one fraction BotuBov® (Botupharma®, Botucatu, Brazil) to final concentration of 60x106 sptz / ml. After dilution, they were loaded into 0.5 mL straws and cooled for 5 hours at 5 different cooling systems: System 1 [S1 cooling curve 0.25ºC / minute to freeze the machine TK 4000® (Tetakon®, Uberaba, Brazil)]; System 2 [S2, cooling curve of 0.5°C / minute to freeze the machine TK 4000®]; System 3 [S3, refrigerator Minitube® (Minitüb®, Tiefenbach, Germany)]; System 4 [S4, BotuTainer® (Botupharma®, Botucatu, Brazil)], and System 5 [S5, Common household refrigerator]. The sperm kinetics was carried out by CASA (computer analysis of semen System - IVOS® version 14) in two stages: in the diluted semen (without glycerol) before refrigeration (S0) and after 5 hours of cooling in the 5 cooling systemas. The variables evaluated were: motility (MOT), progressive motility (PROG) and rapid (RAP). For the evaluation of the plasma and acrosomal membrane integrity, an association of fluorescent probes was used: Propidium Iodide and carboxiflureseína 6-diacetate (Sigma ®, St. Louis, USA). Data were submitted to analysis of variance by proc MIXED (SAS Inst., Cary, USA), and the significance was declared when P <0.05. There was a difference of 5 cooling systems in sperm kinetics and integrity of plasma membrane and acrosome compared with semen evaluated before refrigeration (S0) (P <0.001), but no difference was observed between treatments after cooling. For MOT, the results obtained from each treatment were: S0 = 88.2; S1 = 82.0; S2 = 81.1; S3 = 78.6; S4 = 79.8; and S5 = 78.7 (± 2.60). For PROG: S0 = 70.7; S1 = 54.8; S2 = 56.9; S3 = 56.9; S4 = 57.0; and S5 = 57.2 (± 3.48). For RAP: S0 = 84.6; S1 = 79.4; S2 = 78.9; S3 = 75.7; S4 = 77.2; and S5 = 76.0 (± 2.64). For the lesion of plasma and acrosomal membrane, the results were: S0 = 46.2%; S1 = 52.9%; S2 = 53.5%; S3 = 55.3%; S4 = 54.6%; and S5 = 58.2% (± 6.27%). There is no difference in the sperm kinetics and the plasma and acrosomal integrity of the membrane, after 5 hours by cooling, between the five evaluated refrigeration systems.

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A004 Physiology of Reproduction in Male and Semen Technology

Assessment of scrotal termography and semen quality in buffalo bulls (*Bubalus bubalis*) raised under humid tropical environment

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Keywords: andrology, infrared thermography, water buffalo.

This study aimed assessing the surface temperatures of orbital area and scrotum of buffalo bulls using infrared thermography, monitoring the sperm quality over time and correlating surface temperatures to thermal comfort indexes. The experiment was conducted in humid tropical climate region, from April to August 2013, with maximum daily average of 31.5 ± 0,8ºC, and relative humidity of 81.3 ± 3.8%. Ten water buffalo bulls (Bubalus bubalis, n=10) were maintained in an artificial insemination station (Cebran/UFPA, Castanhal-PA) and were evaluated each 25 days (morning: 6:00-9:00; afternoon: 12:00-15:00). Rectal temperature (RT, ºC) was assessed using thermometry and surface temperatures of orbital area (ORB, ºC) and scrotum (SCR, ºC) were evaluated by infrared thermography. Semen was evaluated for volume, concentration, turbulence, vigor, progressive motility, sperm morphology and plasma membrane integrity, with eosin-nigrosine. Climatic data were continuously monitored and the Temperature and Humidity Index (THI) and the Index of Comfort of Benezra (ICB) were calculated. Variables with non-normal distribution were transformed to logarithmic scale. Analysis of variance was performed by the GLM SAS, version 9.3 (SAS, 2011). It was considered in the model shift (morning and afternoon) and month effects (April to August). For mean comparisons between shifts used F test and for multiple comparison of average monthly was adopted Tukey test. Correlations were calculated using Pearson test. In all analyzes was adopted $P<0.05$. The ICB ranged from 1.96 to 2.25 and significant differences were observed for shifts and over the months ($P<0.05$). The averages of surface temperatures were RT=38.2±0.5°C, ORB=36.1±0.8°C, SCR=33.3±1.1ºC, which exhibited significantly differences for shifts and over the months ($P<0.05$). The gross motility and the sperm vigor were significantly different ($P<0.05$), and a quality decrease during the warmer months and higher THI was detected. The total sperm defects ranged from 17.6±6.2% and 21.2±8.2%, but no significant difference was observed ($P>0.05$). The THI showed positive correlations with ORB (0.72) and ESC (0.41) ($P<0.0001$), while the ICB was positively correlated with ESC (0.25; $P<0.0001$). Negative and significant correlation was found between ITU and sperm plasma membrane integrity (-0.17; $P<0.05$). Therefore, the surface temperatures of buffalo bulls and their semen quality are associated to temperature and humidity changes and suffer interference from climatic variations, justifying the management approaches to provide thermal comfort to animals in order to increase the semen quality.

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A005 Physiology of Reproduction in Male and Semen Technology

Testicular thermolysis ability of "Morada Nova" sheep under heat thermotolerance teste: preliminary results

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Keywords: andrology, hair sheep, thermoregulation.

This research aimed to evaluate thermoregulatory capacity of scrotum, of Morada Nova sheep, subjected to heat tolerance test, using infrared thermography analyses. The study was conducted from January to February 2015 in the experimental unit of Livestock Southeast, São Carlos-SP, subtropical climate region (Cwa Koppen). Seven Morada Nova males were subjected to heat tolerance test (Baccari Junior et al. 1986 Annual Meeting of the Society of Animal Science, 23, p. 316), and yours themolitics answers was evaluated during three distinct periods (T1, T2 and T3), with animal exposure to the sun and shade. In T1, animals were maintained in the shade for two hours (11:00 to 13:00), then they were exposed to direct sunlight for a period of one hour (13:00 to 14:00), featuring T2. In T3, animals returned to the shade, where they remained for an hour (14:00 to 15:00). At end of each period, rectal temperatures were measured (RT-oC) with thermometry. Testicular surfaces temperatures (°C) measured were: dorsal pole temperature (DPT), ventral pole temperature (VPT) and average testicular temperature (ATT). Gradients of temperature between the dorsal and ventral poles were calculated (GDV) and between rectal temperature and average testicular (GRT). These measurements were made with infrared thermal imager (Testo875i, Testo®, Lenzkirch, Germany). Statistical analysis consisted of evaluation of the normality of data, analysis of variance (ANOVA). The effects of time and results were expressed in mean ± SD. Significance level was 5%. Rectal temperatures and testicular surface measured on T2 (RT=39.0±0.3°C, DPT=34.0±1.2ºC, VPT=33.5±1.4°C, ATT=34.0±1.1°C and GRT =4.9 ±1.0 °C) were significantly higher than observed in T1 (RT=38.3±0.3°C, DPT=33.0±1.0ºC, VPT=32.1±1.0ºC, ATT=32.6±0.9ºC and GRT=5.7±0.9ºC) and T3 (RT=38.4±0.3°C, DPT=32.5±1.8°C, VPT=31.4±2.0ºC, ATT=32.1±1.7ºC and GRT=6.3±1.6ºC). Thus, there was no statistical difference between values observed in pre and post challenge. Only gradient between testicular poles (GDV) behaved differently from the others, with statistical difference between T2 (0.48±0.63ºC) and T3 (1.1±0.79ºC). These results demonstrate the thermoregulatory efficiency of Morada Nova sheep, since even after submission to heat stress situation, its surface testicular temperatures were set to baseline standard noted previously challenge. This ensures the maintenance of the physiological temperature gradient between core body and testicles, vital for normal spermatogenesis.

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A006 Physiology of Reproduction in Male and Semen Technology

Cellrox Deep Red® is effective for detecting oxidative stress in bovine sperm: preliminary studies

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Keywords: fluorescent probe, reactive oxygen species, semen.

Reactive oxygen species (ROS) are produced physiologically by sperm cell. Cryopreservation is able to increase this production and it results in presence of antioxidants in composition of majority of extenders. The probe CellROX Deep RED® (Molecular Probes) was validated by our group to identify ROS in ram sperm. However, it has not been efficiently evaluated in bull sperm yet, which is the purpose of the study. For this, commercially available frozen semen from four bulls was used. Two straws of each bull (n=4) were thawed (37°C/30 seconds). The sample was centrifuged at 500 g/10 minutes to remove the supernatant (extender sperm free), stored at 5°C. The precipitate was suspended in 200 µL of Tyrode's albumin lactate pyruvate (TALP) and added to 50 µL of iron sulfate and 50 µL of ascorbic acid kept at 37°C/90 minutes maintained with open tube cap for oxidative stress induction. The sample was centrifuged at 500 g/10 minutes. The supernatant was removed and the precipitate was suspended in 300 µL of TALP. The concentration was adjusted to $20x10^6$ sperm/mL, diluting one sample in TALP (control group), and another in the stored extender. It was performed that to evaluate if the probe would be able to identify sperm with ROS in control group, since in extender group probably it would be observe a little level of oxidative stress because of antioxidants proprieties of extender. Aliquots of 200 µL of the samples (control x extender) were added with 4 µL of CellROX Deep RED® 1 mM and 1µl of Hoescht 33342 0.5 mg/mL (Molecular Probes) and incubated at 37°C/30 minutes. The samples were centrifuged at 5000 g/5 minutes, the supernatant was removed and the samples were suspended in 200 µL of TALP. It was prepared a humid chamber with 4 µL of the samples and 200 cells per slide were counted. The cells were classified as absent or few presence of ROS (FEW), moderate level of ROS (MOD) and intense level of ROS (INT). FEW and INT variables were transformed and subjected to analysis of variance (ANOVA). For the variable MOD, an evaluation by nonparametric statistics was made. It was used SAS software (SAS Institute Inc., 2004) and the significance level was 5%. After the stress induction, control group showed a reduction ($p=0.005$) in FEW cells (5±3.84%) when compared to extender group (74.87±18.54%). There was no difference ($p=0.24$) between control group (68.87±4.14%) and extender group (24.50±18.26%) for MOD cells. However, control group (26.12±1.86%) showed higher ($p<0.0001$) INT amount of cells than the extender group (0.62±0.31%). Thus, it can be concluded that the CellROX® probe is able to identify ROS in bovine spermatozoa and that the extender is capable of neutralizing these species. However, more studies are being done by our group to confirm the efficiency of the ROS evaluation in bull criopreserved sperm.

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A007 Physiology of Reproduction in Male and Semen Technology

Single layer centrifugation improves stallion sperm motility after storage at 15ºC

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Keywords: cooling, coloid, ejaculate.

Cooling of stallion semen for transport and subsequent artificial insemination has become widespread in equine reproduction. Cooled storage prolongs the lifespan of spermatozoa, however, this condition can also induce irreversible damages to structural integrity, biochemistry and biophysics of the sperm cell (Aurich C, Anim Repro Sci, 107:268-275, 2008). In addition, there is considerable variability in sperm survival of individual stallions during the process. Single layer centrifugation (SLC) is a colloid centrifugation technique designed to select spermatozoa with good motility, membrane integrity, normal morphology and intact chromatin from the rest of the ejaculate (Morrell JM & Rodriguez-Martinez H, Open Andro J, 1:1–9, 2009), improving sperm quality. The aim of this experiment was to evaluate the effect of SLC in stallion sperm motility after cooled storage at 15ºC for 8 hours. During reproductive season and after two days of sexual arrest, ejaculates were collected from ten Crioulo breed stallions (n=10) with artificial vagina and an estrus mare. After collection, the ejaculate was filtered and diluted with commercial semen extender (EquiPlus®, Minitüb GmbH, Tiefenbach – Germany) to $50x10^6$ sptz/mL. Semen samples were refrigerated for 8 hours at 15ºC and then submitted to two treatments: Conventional centrifugation (600G x 20') and SLC (Androcoll Equine®, Minitüb GmbH, Tiefenbach – Germany) (300G x 20'). In both protocols, the pellet was ressuspended with the same extender. Progressive motility (%) was evaluated by Computer Asssited Sperm Analyzer (AndroVision® Minitüb GmbH, Tiefenbach – Germany) in three moments: 1) after collection and dilution, 2) after 8h of cooling (pre-centrifugation) and 3) after centrifugation and ressuspension (post-centrifugation). Descriptive statistic and mean comparison by Kruskal-Wallis test were performed in the program Statistix9® ($p<0.05$). The semen diluted had shown a 68.7±4.2 mean of percentage progressive motility. Along the cooled storage period, the sperm motility mean dropped significantly ($p<0.05$) to 48.7±6.2 (Conventional centrifugation) and 49.9±6.0 (SLC). The sperm percentage motility after conventional centrifugation and ressuspension (45.2±5.6) had no difference in comparison with the previous moment, while the SLC treatment resulted in 58.5±5.7. The mean of sperm motility after SLC was significantly higher ($p<0.05$) when compared with the moment pre-centrifugation . There was no statistical difference between the moment post-SLC when compared to the initial motility measured prior cooled storage demonstrating the efficiency of this method to improve the semen quality selecting the better sperm cells. Semen samples submitted to SLC had 29.4% higher progressive motility than the sample with normal centrifugation. It is suggested that these semen samples could be used for subsequently artificial insemination of mares or posterior methods to increase its shelf life. In the present experiment, it is demonstrated the beneficial effect in progressive motility of SLC after cooled storage at 15ºC for a short period of time.

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A008 Physiology of Reproduction in Male and Semen Technology

Comparison of blood flow on testicular artery, hormone dosage and semen quality between texel and "Santa Ines" sheep

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Keywords: hormonal dosage, sheep, spectral Doppler.

The aims of this study were to evaluate and compare testicular parameters, sperm quality and hormonal dosage between Texel and Santa Inês sheep. For this, 25 healthy animals (12 Texel and 13 Santa Ines) aged between 2 to 4 years old were used, from the Department of Animal Reproduction and Veterinary Radiology of Sao Paulo State University (UNESP – Botucatu). Testicular morphometry was initially performed (length - LEN and width - WID in centimeters) and then the spectral doppler ultrasound examination of testicular artery (pulsatility index - PI and resistance index - RI) was evaluated in the spermatic cord. Subsequently a sample of blood from each animal was collected in the morning by puncture of the external jugular vein were collected for access the testosterone dosage (DT, mg/dL) and then the semen was collected by electroejaculation. The sperm kinetic parameters were analyzed by computerized method CASA (TM% total motility, %PM - progressive motility, µm/s VAP – average speed path, µm/s VCL - curvilinear velocity, µm/s VSL - progressive linear speed, %RAP - rapid sperm) and the plasma membrane integrity (MPI) was analyzed by epifluorescence microscopy. The testosterone dosage was access by kits and the analysis performed by radioimmunoassay. The data generated were evaluated by Student´s t-test and differences were considered significant at $p<0.05$. The mean values and standard deviations found for the evaluated parameters for Texel and Santa Ines breeds are placed following, respectively: TM (81±7.4 and 84±17.8); PM (57.4±10.1 and 62.1±17.4); VAP (130.4±19.6 and 131.6±18.3); VCL (195.2±38.1 and 181.0±22.3); VSL (123.4±29.4 and 111.6±8.9); RAP (70.0±10.2 and 74.4±18.3); MPI (64.8±11.4 and 61.2±12.9); LEN left testicle (9±1.11 and 9.5±1.2); WID left testicle (6.2±0.6 and 6.3±0.5); LEN right testicle (9.1±0.9 and 9.7±1.2); WID right testicle (6.1±0.7 and 6.2±0.5); DT (1.08±0.65 and 2.97 5.6); PI left testicle (0.99±0.27 and 1.16±0.33); RI left testicle (0.63±0.11 and 0.68±0.12); PI right testicle (1.16±0.46 and 1.17±0.47) and RI right testicle (0.67±0.17 and 0.68±0.16). In conclusion there was no difference in any of the parameters evaluated between the sheep breeds.

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A009 Physiology of Reproduction in Male and Semen Technology

Different *in vitro* sperm challenge and its relationship with *in vivo* bull fertility

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Keywords: Brangus, fertility, laboratory sperm challenge.

The aim of this work was to challenge the laboratory quality of thawed semen and to compare the in vitro results with in vivo semen fertility. Frozen-thawed semen of 4 different batches from the same bull, which were previously used in a TAI program were used for insemination of 332 Brangus cows. For laboratory experiment, three repetitions of each batche was performed. For each semen dose, the following procedure was accomplished: initially, the semen sample was thawed at 37°C for 30 sec (control), sperm motility was assessed by CASA and plasma membrane integrity was evaluated by propidium iodide fluorescent probe. Then, an aliquot of 150 µL of the sample was incubated in a water bath at 45°C for 40 min (thermal challenge group; GDT) and another aliquot of 150 µL of the sample was centrifuged at 500 x g (Percoll gradient 45%/90%) for 15 min (centrifugation challenge group; GDC). The centrifuged semen was also subjected to another thermal challenge, being incubated (water bath) at 45°C for 40 min (centrifugation + thermal challenge group; GCDT). At the end of each challenge (GDT, GDC and GCDT), the same laboratory tests used for control group were repeated. The field data were analyzed by GLIMMIX of SAS and laboratory data by analysis of variance in GraphPad INSTAT. Significance level of 5% was established. No difference ($P>0.05$) between AI technitian, BCS or batches (B) was observed for conception rate (CR). The following CR were observed for each batch: B1 = 48.9% (44/90); B2 = 44.2% (23/52); B3 = 55.5% (40/72); B4 = 43.2% (51/118). Although no statistical difference was observed between batches, numerically higher CR was observed for B3 compared to B4. According to CASA results, it was interesting to note that B4 was the batch that presented lower ($P<0.05$) percentages of Progressive Motility (PM) both after thawing (control: 47.2 ± 8.5) and after all sperm challenges (GDT: 40.0 ± 4.6; GDC: 45.7 ± 7.3; GCDT: 4.7 ± 7.2) compared to B3 (control: 63.0 ± 5.3; GDT: 56.0 ± 1.7; GDC: 64.2 ± 12.5; GCDT: 7.7 ± 3.8). In addition, while B3 and B4 demonstrated similar percentage of plasma membrane integrity (MPI) in control (T3 = 66.7 ± 1.3 and T4 = 65.2 ± 3.3), the semen of B3 demonstrated higher ($P<0.05$) percentage of MPI (37.2 ± 2.5) than B4 (26.7 ± 3.3) after passing through the greatest challenge of this in vitro experiment (GCDT). According to the results, it was concluded that the semen of batch 3 was the most resistant to the proposed laboratory challenges, especially when compared to batch 4. Therefore, the present study suggests that to submit seminal samples to a laboratory challenge before to perform an in vivo semen quality assessment seems to be an interesting alternative for define semen batches that may present greater reproductive performance of field fertility.

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A010 Physiology of Reproduction in Male and Semen Technology

Viability and longevity of bovine epididymal spermatozoa after cryopreservation

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Keywords: bovine, kinects, semen.

The recovery of epididymal spermatozoa (EP) and its use in assisted reproductive techniques have an important role in multiplication of genetic material from bulls that died suddenly and/or have acquired reproductive failure. However, to maximize the *in vivo* and *in vitro* use, a better knowledge about the physiologic behavior of those spermatozoa is necessary. The aim of this study was to characterize the viability and longevity of cryopreserved sperm recovery of epididymal tail, for 24 h. Epididymis (EP) and ejaculated (EJ) sperm were recovered from the same bulls of Gir breed (n=7) and were cryopreserved. Three groups were used: EJ, EP and EP that were exposed for 10 min at 39ºC in a pool of seminal plasma (EPP). The three sperm groups were selected in Percoll gradient 45:90% and were incubated at 39ºC for 24 h in SP-TALP buffer medium in a final concentration of $2x10^6$ spermatozoa/ml. Sperm samples from the three groups (EJ, EP and EPP) were removed at 0, 3, 6 and 24 h of incubation and were evaluated for total (TM) and progressive motility (PM) (CASA), morphology (phase contrast), capacitation (chlortetracycline-CTC), plasma membrane integrity, assessed with propidiun iodide (PI) and 6-carboxyfluorescein diacetate (C-FDA) and acrosomal integrity technique Peanut agglutinin (PNA conjugated to FITC). Data were analyzed by GLIMMIX procedure using SAS program ($P \leq 0.05$). At 6 h of incubation EJ group showed a bigger decrease on MT (33.9 ±8.8), MP (EJ 23.3± 8.1), IMP (16.4±3.4), IA (19.9±3.2), and percentage of non capacitated sperm (70.3±3.7) in relation to EP and EPP groups. The EP and EPP groups, at 6 h of incubation showed MT (56.7±9.3 and 40.6±9.2), MP (47±9.6 and 35±9.2), IMP (29±4.3 and 28.2±4.2), IA (31.6±3.8 and 31.6±3.8), and percentage of non capacitate sperm (80. 9±3.5 and 81.0±3.5) similar to those observed at 0h. At 24 h of incubation, all groups presented similar MT (EJ=0.5±1.4, EP=9.8±5.7; EPP=10.6±5.9), MP (EJ=0.4±1.3; EP=6.5±4.9 and EPP=8.2±5.5), IMP (EJ=6.7±2.3; EP=10.3±2.8 and EPP 10.7±2.8) and IA (EJ=7.1±2.0; EP=10.3±2.4; EPP=11. 1±2.5). However, the percentages of non capacitate sperm was lower on the EJ group (68.0±4.6) than on the other groups, EP (76.0±3.8) and EPP (80.5±3.5). It can be concluded that after thawing, epididymis sperm were able to maintain their quality for a longer period than the ejaculated, suggesting they have higher cryo resistance than the ejaculated sperm. In addition, exposition of EP to seminal plasma did not affected EP viability and/or longevity.

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A011 Physiology of Reproduction in Male and Semen Technology

Effects of cryopreservation on motility of Nordestino breed stallion sperm

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Keywords: CASA, equine, kinematic.

Studies show that the racial factor directly affects the sperm cell resistance to cryopreservation. In addition, in some stallions, the low quality of frozen semen appears to be related to the presence of glycerol in freezing media (Alvarenga; Papa 2011, Spermova 1:7-10). Although Nordestino breed is essential in the Caatinga, there are few studies on cryopreservation of semen of stallions of this breed. This study aimed to analyze the motility of the sperm of stallions of the Northeastern race after cryopreservation. This study was approved by the CEDEP of UNIVASF (Protocol nr. 0006/161012). Nineteen ejaculates used two stallions (N = 10 and N = 9 ejaculates, respectively) were collected by artificial vagina and female in oestrus. The ejaculates showed milky white-gray coloring, sui generis odor, and the total sperm motility (TM%) of 80.91 ± 9.29 and progressive (PM%) of 59.24 ± 11.45. After collection, the samples were diluted (1:1) with Botu-Semen® (Botupharma Botucatu, SP), centrifuged in 50 ml tubes at 2200 rpm for 15 min and the pellet resuspended with Botu-crio® (Botupharma, Botucatu, SP) to determine sperm concentration using the photometer Spermacue® (Minitube, Berlin, Germany). After this, the semen was packaged in 0.5 ml straws and kept at 5°C for 90 min and then packaged into 0.5 ml straws, frozen in liquid nitrogen vapor for 20 min before being plunged into liquid nitrogen for storage. Semen was thawed in a water bath at 37°C for 30 s. The TM and PM were determined using the CASA® (Minitube, Germany, Berlin). Aliquots (8 µL) of thawed semen were removed, placed between slide and cover slip, pre-heated to 37°C, and evaluated for sperm motility. Data were analyzed using ANOVA and SNK test ($P<0.05$; SAEG, UFV 1997). The TM and PM of sperm after thawed was 34.08 ± 12.57 and 20.53 ± 12.82, respectively. Preliminary results indicate that the Stallions Nordestino breed analyzed have good semen freezability and keep the parameters of total and progressive sperm motility.

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A012 Physiology of Reproduction in Male and Semen Technology

The effect of heat shock on bovine sperm motility and cytoskeleton organization

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Keywords: heat shock, immunofluorescence, sperm.

Bull heat stress increases scrotal and testicular temperature, reduces sperm quality, motility and fertilization ability. Sperm changes in motility are directly associated with organization of cytoskeletal proteins. Therefore, the objective of this study was to determine the effect of heat shock on bovine sperm motility and cytoskeleton organization. A pool of three Bos indicus semen straws was used for each replicate (N = 6 replicates). Semen straws were purified on Percoll gradient. Sperm sample ($1x10^6$ spermatozoa/ml) was subjected to motility evaluation and immunofluorescence for localization of microtubules and the molecular motor dynein immediately after Percoll gradient (0 h Control) and after SP-TALP incubation at Control (38.5°C) and Heat Shock (41°C) for 4 hours. Sperm motility was assessed every hour during the incubation period. Samples of the 0 hour control and after 4 hours incubation were processed with anti-α-tubulin mouse monoclonal IgG, anti-cytoplasmic dynein rabbit polyclonal IgG, Alexa Fluor 488® goat mouse IgG, Alexa Fluor 555® goat mouse IgG and Hoechst 33342 for sperm tubulin, dynein and DNA localization. Tubulin (N= 600 sperm/treatment) and dynein (N = 300 sperm/treatment) morphological localization and pixel fluorescence intensity was determined with Image J software version 1,49j. Non-parametric data were analyzed using the Wilcoxon test of the statistical package SAS. Zero hour control (not incubated) sperm motility was higher than all the other groups (73.12 ± 3.6%, $P < 0.05$). Sperm incubation at 38.5°C affected ($P < 0.05$) sperm motility at different incubation times (68.12 ± 3.6%, 53.75 ± 3.6%, 41.25 ± 3.6% and 27.12 ± 3.6% for 1, 2, 3 and 4 hours, respectively). Similarly, incubation of spermatozoa at 41°C ($P < 0.05$) reduced sperm motility over time (52.5 ± 3.6%, 43.75 ± 3.6%, 20.62 ± 3.6 % and 3.62 ± 3 6% for 1, 2, 3 and 4 hours, respectively). Heat shock of 41°C reduced ($P < 0.05$) sperm motility as compared to control 38.5°C at all incubation times. Immunofluorescence indicated that tubulin was localized at the sperm tail insertion and along the tail. Tubulin pixel fluorescence intensity increased ($P < 0.01$) with incubation and temperature (13.6 ± 0.29, 15.2 ± 0.28, 15.4 ± 0.28 arbitrary units (AU) for 0 hour control, 38.5°C and 41°C, respectively). The molecular motor dynein was localized along all the sperm cell. Similarly, dynein pixel fluorescence intensity increased ($P < 0.0001$) with incubation (4.2 ± 0.5, 26.8 ± 0.5, 26.2 ± 0.5 AU for 0 hour, 38.5°C and 41°C, respectively). In conclusion, incubation and elevated temperature reduced sperm motility and affected the pattern of cytoskeletal proteins organization.

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A013 Physiology of Reproduction in Male and Semen Technology

Effects of plasma testosterone and total protein of seminal plasma on the sperm parameters of semen donors Nelore bulls

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Keywords: seminal plasma, testosterone, total protein.

Researches aiming to relate testosterone concentration (T) and protein fractions of seminal plasma (SP) are common, mainly those related to sperm production and freezability, respectively. However, few studies have sought to establish an association between T or SP total protein (TP) and spermatic variables usually used to evaluate sperm quality in Semen Collecting and Processing Center. Thus the goal of this study was to verify the influence of plasmatic T and TP of SP on the sperm parameters from semen donors bulls. Forty ejaculates from 8 Nelore bulls were collected by artificial vagina. The sperm samples were examined for motility (M, %), vigour (V, 0-5), concentration (C; spermatozoa/mL) and sperm morphology (%; major defects [MD], minor defects, [mD] and normal [N], before freezing. The SP was obtained by semen centrifugation at 700 x g/10 min. The blood (five samples per animal) was collected immediately after semen collection through the jugular vein puncture. Samples of T and TP (kept at -86o C until using) were measured using commercial kits (DPC- Diagnostic Products Co®., EUA; Pierce, EUA, respectively), according to manufacturer's instructions . The results of T and TP were expressed in values of mean±standard deviation. The correlation coefficient of Spearman was used to verify possible correlations between spermatic variables and T or TP, with $P<0.05$ taken as significant. Data of T and TP were 684.46±212.01 and 37.30±1.7, respectively. Significant correlations just were observed between TP and spermatic variables, such as: vigour ($r = 0.3818$; $p<0.015$), MD (-0.5004; $p<0.001$), mD ($r = -0.3240$; $p<0.041$) e N ($r = 0.443$; $p<0.005$). On the other hand, there was tendency of correlation between TP and M ($r = 0.266$; $p<0.08$). However, no significant correlations were found between the spermatic variables and T. In conclusion, under these experimental conditions, the concentration of TP of SP was effective in determining best results of sperm quality of important variables (V, MD, mD, and N), suggesting that TP acts as a modulator agent for these variables, through a mechanism not yet established. Nerveless, other investigations with the largest number of animals need to be carried out to confirm these findings, which might lead to the determination of useful marker to monitor sperm quality of semen donors Nelore bulls.

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A014 Physiology of Reproduction in Male and Semen Technology

Efectiveness of distilled water for hipoosmotic swelling test in cryopreserved ovine semen

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Keywords: frozen sêmen, hipoosmotic swelling test, ovine.

The hipoosmotic swelling test (HOST) is interesting for the evaluation of functional integrity of sperm plasma membrane, also being an affordable and easy method, especially when using distilled water as hypoosmotic environment. The HOST has been used as evaluation protocol of sperm viability of many species; however, the use of distiller water as hipoosmotic solution was not standardized for ovines. Besides, the effectiveness of distilled water with traditional hipoosmotic solutions, such as citrate-fructose, has not been compared. The aim of this study was to analyze the reactivity of post-thaw sperm cells of Santa Ines and Dorper rams in distilled water and citrate-frutose (100 mOsmol/L) hipoosmotic solution, both at different dilution rates. Thirty semen samples (20y Santa Ines and 10 Dorper rams) were used. After the thawing, the kinetic sperm parameters were analyzed (total motility-TM, progressive motility-PM and sperm vigor-VIG). Aliquots were collected for the supravital test with dye eosin (EOS); the sperm morphology was analyzed and the percentage of bent tails (BT) calculated. The HOST followed the dilution: one part of semen to 10 (HOST G1), 20 (HOST G2) and 50 (HOST G3) parts of solution. The same proportions were maintained for distilled water as following: one part of semen to 10 (HOST G4), 20 (HOST G5) and 50 (HOST G6) parts of solution. The percentage of HOST-reactive spermatozoa was determined by subtracting the percentage of spermatozoa with HOST-induced bent tails (BT) from the BT obtained right after thawing. These evaluations were carried out in phase contrast microscopy (1000x) and one hundred cells were analyzed per semen sample. All the statistical analysis were performed by using the SAS software, version 5.0 (1996) (MEANS and GLM Procedure – SNK test, with $P<0.05$). The post-thawing values were for total motility: 63.3%; progressive motility: 58.3%; vigor: 3.1; bent tails: 18.4%; sperm viability by the supravital: 42.9% and HOST: 32.4%. The averages observed in the hipoosmotic test were: HOST G1 (36.1%); HOST G2 (34.5%); HOST G3 (34.6%); HOST G4 (27.2%); HOST G5 (30.0%) and HOST G6 (32.2). Despite the numerical variations among the HOST groups, the rates did not differ ($P>0.05$) significantly. The findings demonstrate that the HOST with distilled water is effective and can be used for the evaluation of post-thawing ovine sperm viability.

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A015 Physiology of Reproduction in Male and Semen Technology

Estimating the fertilizing ability of collared peccaries (*Pecari tajacu*) sperm by analyzing its interactions with swine oocytes

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Keywords: semen, *Tayassu tajacu*, wild pig.

Conventional tests that evaluate sperm quality do not have the ability to measure the fertilizing potential of a sample. The aims of this study were to evaluate the binding capacity of collared peccaries (Pecari tajacu) sperm using the heterologous in vitro interaction test with swine oocytes from antral follicles, and stablish the relations between sperm parameters and the binding test. Thus, a total of 11 ejaculates from adult individuals collected by electroejaculation was evaluated for motility, vigor, viability, normal morphology, kinetic motility parameters by computerized assisted semen analysis (CASA), membrane functionality and integrity. Moreover, 11 samples were analyzed by the in vitro interaction test using swine oocytes at 38.5°C and 5% CO2 for 18 h. After this period, the oocytes were washed and labeled with Hoechst 33258 (10 µg/mL) and visualized by fluorescence microscopy. The estimated fertilizing capacity was analyzed according to the number of bound sperm and/or penetrated the zona pellucida. All the data were expressed as mean ± SD and a simple linear regression model was used to identify associations between sperm-oocyte interactions (dependent variables) and sperm parameters (independent variables). Thus, the mean values for semen parameters evaluated by conventional analysis and CASA were as expected for the species. In the in vitro interaction test, we verified that all the swine oocytes (100%) presented bound sperm to zona pellucida, but only 19.85 ± 5.5% oocytes presented penetrated sperm. Additionally, an average of 39.4 ± 4.6 bound sperm/oocyte and 2.5 ± 0.7 penetrated sperm/oocyte were found. Probably, the composition of the zona pellucida of swine oocytes is similar to the peccaries, thus suggesting its use as heterologous substrate for the evaluations of sperm penetration capability for peccary. Among the sperm parameters, only the straightness rating – STR presented association to the number of bound sperm ($R = 61.7\%$; $P < 0.05$). Such parameter is related to progressive sperm, indicating the hyperactivation, and it is related to the fertility. In conclusion, the *in vitro* interaction test at using swine oocytes do not present marked relations to sperm parameters currently evaluated in collared peccaries. Further studies are needed to enable the use of heterologous substrates as accurate indicator of fertility for the species.

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A016 Physiology of Reproduction in Male and Semen Technology

Seminal quality comparative study between pets and working animals of the Australian Cattle Dog breed

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Keywords: Australian Cattle Dog, dog, semen.

The objective of this study was to evaluate seminal characteristics in dogs of the Australian Cattle Dog breed. The animals were divided into two groups: Group I (GI) pets who do not perform regular physical activity (N1 = 4) and Group II (GII) animals used daily in animal husbandry work (grazing) on farms (N2 = 3). We evaluated data scrotal circumference and seminal characteristics of seven dogs, evaluating three ejaculates from each animal (N = 21 ejaculates). The collection of the semen was performed by digital manipulation; being only used the second fraction of the ejaculate for evaluation. The ejaculates were evaluated for macroscopic characteristics: volume (VOL) and microscopic: progressive motility (MOT), vigor (VIG), concentration (CONC) and morphology (MORF). For testis size were carried out scrotal circumference measurements (CE), right testicle long (CTD) and left (CTE) and wide right testicle (LTD) and left (LTE), with the help of a tape measure and calipers, respectively. The means and standard deviations for the variables weight and age of the animals were 22.18 ± 3.43 kg and 4.57 ± 2.63 years, respectively. For the evaluation of variables related to semen characteristics we used the statistical model that looked at the group of fixed effects (GI vs. GII), and random effects of animal and residual. Analyses were performed considering structure of repeated measures in the same animals, by using the PROC MIXED SAS (SAS INC, 2004). For CONC variable, data were submitted to logarithmic transformation. For the EC variables, CTD, CTE, LTD and LTE the average estimates, and the standard deviations found were respectively 6.31 ± 0.95 cm, 1.75 ± 0.27, 1.85 ± 0.25, 2.30 ± 0.36 and 2.64 ± 0.57 cm. For the variables related to seminal quality, average estimates were observed and standard deviation: VOL, 1.11 ± 0.21 and 2.01 ± 0.25 mL; MOT, 82.08 ± 4.32 and 73.33 ± 4.99%; VIG, 4.00 ± 0.15 and 3.22 ± 0.19; Pathol, 7.08 ± 0.91 and 6.1 ± 1.05%, and CONC, 8.43 ± 0.09 and 8.36 ± 0.11 sptz/ejaculate. The analysis of variance revealed a significant effect ($P < 0.05$) for the VOL (GI: 1.12 ± 0.22^{b}; GII: 2.01 ± 0.25^{a} mL; $P = 0.0441$) and VIG (GI: 4.0 ± 0.17^{a}; GII: 3.22 ± 0.19^{b}; $P = 0.03$) variables. The results of this study suggest that although the two groups present semen quality, companion animals (GI) exhibited higher values for vigor compared to animals used for work (GII). As for the VOL variable, the working animals (GII) presented higher values than pets (GI).

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A017 Physiology of Reproduction in Male and Semen Technology

Significance of morphofunctional semen evaluation of buffalo bulls used for timed artificial insemination

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Keywords: buffaloes, cryopreservation, functional tests.

Around 50% of sperm are lost during bovine semen cryopreservation process (Arruda, R. P. Biotecnologia da reprodução em bovinos. 1º Simpósio Internacional de Reprodução Animal Aplicada, p.166-179). In such context, the development of laboratory tests aiming to predict more accurately the performance of cryopreserved semen has been target of field researches in AI programs (Arruda, R. L. Ver. Bras. Reprod. Anim., v.34, p.168-184, 2010). The present work aimed to demonstrate the influence of morphological and functional semen analysis for the success of fixed time artificial insemination programs (FTAI) in buffaloes using cryopreserved semen. One hundred and five buffaloes [59.1±2.4 days post-partum and average body score condition of 3.7±0.0 (1-5)] were synchronized to TAI at a random day of the estrous cycle (D0; 16:00). All the buffaloes were treated with a P4 intravaginal releasing device (1g progesterone; Sincrogest®, Ourofino) and 2.0 mg of estradiol benzoate im (Sincrodiol®, Ourofino). On D9 (16:00), females received 0.53 mg im of PGF2α (Cloprostenol, Sincrocio®, Ourofino) and 400 IU eCG im (Novormon®, MSD Animal Health), followed by the removal of progesterone device. On D11 (16:00), 10 µg of Buserelin Acetate (GnRH, Sincroforte®, Ourofino) were administered im. The TAI was performed 16 hours after the application of GnRH (D12; 8:00). Sixty four (64) semen straws of bull 1 and 41 straws of bull 2, from the same batches, were used in the same TAI protocol. Bulls were selected according to the following criteria: motility>50%, vigor>3, concentration higher than 10 million sperm/straw, total defects<30%. Although approved by such criteria, there was a difference of 8.5% between pregnancy rates of both bulls (bull 1: 71.9; bull 2: 63.4). Three semen samples of each batch were thawed and subjected to functional semen analysis (ie, plasma membrane integrity - Eosin-Nigrosine; acrosome integrity - Fast-Green/Rose Bengal; mitochondrial activity – Diaminobenzidine; DNA fragmentation - SCSA and lipid peroxidation - TBARS). Despite the lower percentage of motile cells (bull 1: 56.7±3.3% vs. bull 2: 65.0±2.9%), the higher fertility bull showed similar number of mobile sperm per straw (18.5% vs. 18.4%). However, the higher fertility bull showed a lower percentage of major defects when compared to lower fertility bull (7.0±0.6% and 20.3±0.9, respectively). There were also a higher percentage of cells with intact plasma acrosome membranes in the highest fertility bull (bull1: 80.7±4.1% and 95.3±0.3%; bull 2: 63.7±2.4% and 76.7±0.9%, respectively). No differences were found on mitochondrial activity, DNA fragmentation or lipid peroxidation. Preliminary results indicate that together physical analysis, sperm morphological and functional tests may be essential for the assessment of post-thaw fertility in buffalo TAI programs.

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A018 Physiology of Reproduction in Male and Semen Technology

The influence of different methods of frozen-thawed ovine spermatozoa selection on sperm capacitation and viability after incubation

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Keywords: ram, sperm longevity, sperm selection.

Sperm capacitation is an essential event for fertilization; however, it decreases the sperm lifespan and viability. The aim of this study was to evaluate the effects of four sperm selection techniques on sperm capacitation and viability after incubation. A pool of frozen-thawed sperm from 10 Santa Inês rams was used. The samples were submitted to one of the following sperm selection techniques: sperm washing, Percoll gradient, mini-Percoll gradient, Swim-up and control group. At mini-Percoll technique, was used 400 microliters of 90% and 45% gradients and a centrifugation at 500 xg for 5 minutes. In Percoll, was used 1 mL of each gradient and a centrifugation at 700 xg for 10 minutos. During Swim-up, the sperm was incubated in 1 ml of SPERM-TALP for 45 minutos in humidified atmosphere at 37.5°C. Finaly, at sperm washing the sample suffered centrifugation at 300 xg for 8 minutes, using SPERM-TALP. At the end of each treatment, the selected spermatozoa were incubated at 37°C for 1 h, 2 h, and 3 h. Viability was assessed using acridine orange-propidium iodide combination by computer-assisted sperm analysis. Capacitation status was evaluated using chlortetracycline staining and observed under epifluorescence microscopy. Data were analyzed by ANOVA, followed by Tukey test ($P<0.05$). After 3 h of incubation, the capacitated sperm was decreased ($P<0.05$) in all treatments. The capacitated sperm rate was similar ($P>0.05$) among Percoll (36%), mini-Percoll (34%) and Swim-up (30%), and were lower ($P<0.05$) than control group (47%) and sperm washing (41%), regardless of the time of incubation. The non-capacitated sperm percentage was higher ($P<0.05$) at 0 h (12%) and decreased after 3 h (1.5%), in all treatments. Regarding to acrosome reacted cells, there was an interaction ($P<0.05$) between incubation and sperm selection treatment. The acrosome reacted spermatozoa showed a lower percentage ($P<0.05$) at 0 h (50%) and 1 h (53%) and higher after 3 h (64%). Percoll and mini-Percoll were higher about acrosome reacted spermatozoa ($P<0.05$; 60% vs. 61%), whereas control group was the lowest (49%). There was an interaction ($P<0.05$) between incubation and treatment in sperm viability. Viability assays revealed that 0 h resulted in a higher rate (17.5%; $P<0.05$) of membrane integrity, after all treatments. Swim-up treatment showed a higher membrane integrity rate (17.4%; $P<0.05$), regardless of time of incubation. In conclusion, the incubation affects the capacitation status and viability of frozen-thawed ovine sperm. Sperm selection increases the acrosome reacted cells rate and Swim-up allows better viability during incubation.

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A019 Physiology of Reproduction in Male and Semen Technology

In vitro production of bovine embryos using frozen semen with or without the presence of seminal plasma

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Keywords: centrifugation, filtration, Nellore.

Seminal plasma, that mixes with sperm in the ejaculate, and serves as a means of transportation to the female genital tract, has been described as beneficial and harmful to the spermatozoa. There are reports of negative influence of seminal plasma on the storage of semen, due to components harmful to sperm viability. An alternative to reduce the concentrations of seminal plasma of the ejaculate is its semen centrifugation or filtration. However, several studies have reported apparent injury to bovine sperm damaging fertilization by the method of centrifugation. The objective of this study was to evaluate rate of bovine embryos produced in vitro (IVP) using frozen semen with or without the presence of seminal plasma. Semen used for IVP was obtained from 31 Nelore bulls collected by electroejaculation. The semen sample was divided into three equal aliquots and the treatments performed as Campanholi et al. (VII Congresso Interinstitucional de Iniciação Científica (CIIC), Campinas, São Paulo, Brasil, 2013, p.1-8). Treatment 1 (Conventional) constitutes the dilution of semen in the traditional freezing to the final concentration of $60x10^6$ spz/mL with extender BotuBov® (BotuPharma®, Botucatu, Brasil). Treatment T2 (Centrifuged) involved the centrifuging for 10 minutes at 600xg (2200 rpm) for removal of seminal plasma. Treatment T3 (Filter) was performed by the Sperm Filter® (filtration device; BotuPharma®, Botucatu, Brasil). After centrifugation and filtration of semen, spermatozoa were resuspended with extender BotuBov® at the same concentration of T1. After treatments semen was packaged at room temperature in 0.5 mL straws and frozen using the machine TK 4000® (Tetakon®, Uberaba, Brasil). Bovine oocytes to IVP were obtained from follicular aspiration from slaughterhouse ovaries. The statistical analyzes were performed in SAS PROC GLM, using 5% significance. No differences among treatments were detected in cleavage rate, T1=82.1 ± 0.83% (3748/4570), T2=82.05 ± 0.83% (3719/4535) and T3=84.01 ± 0.83% (3786/4507). The rate of embryos evaluated on D7 was higher ($P<0.001$) in T1 (31.30 ± 1.07%, 1430/4570) and T3 (32.3 ± 1.07%, 1476/4570) when compared to the treatment T2 (26.55 ± 1.07%, 1204/4535). The T3 (24.0 ± 1.05%; 1082/4570) treatment had higher ($P<0.001$) hatched blastocyst rate than T2 (18.03 ± 1.05%; 854/4535) and rate similar to T1 (21.22 ± 1.05%; 970/4570). Thereat, seminal plasma removal using SpermFilter® not changed the rate of embryos on days D7 and D9 when compared with conventional frozen semen.

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A020 Physiology of Reproduction in Male and Semen Technology

Deep cervical insemination by cervical catheterization in dairy ewes

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Keywords: ovine, cervical catheterization, clamping

The transcervical artificial insemination (AI) technique is limited in ewes because the cervical rings morphology prevents or impairs the introduction of the AI pipette. The positive correlation between the cervical penetration ease at AI and pregnancy rate justifies the development of techniques that allows a deeper deposition of semen into the cervix. This study aimed to compare the pregnancy rates after superficial versus deep cervical AI. The deep AI was performed due by clamping and traction of the vaginal fornix, and the catheterization of the cervix using the semen applicator (Alta genética®). Fifty ewes were synchronized using vaginal progesterone pessaries and injection of 350 IU eCG, combined with 0.125 mg of prostaglandin at pessary removal (day 12). Estrus detection started 12 h after pessary removal, and AI was performed 12 h after estrus detection, being the ewes randomly allocated into each of the 2 experimental groups. Twenty-seven ewes were inseminated using the superficial cervical AI technique, and 23 were bred by the deep cervical technique (only considered if the catheter would pass through the third cervical ring). For both techniques, the female hindquarters were raised at a 45º angle in relation to the ground. With the aid of a vaginal speculum the cervix was located, and the semen was deposited either in its entrance (superficial cervical AI) or after the third cervical ring, in this case using topical anesthesia to clump the fornix and traction the cervix (deep cervical AI). The semen used was collected from 4 rams (Lacaune and Milchshaff breed), diluted 1 + 3 in Tris egg yolk, loaded in 0.25 mL straws and cooled at 5 °C. Starting from room temperature, the semen was cooled at a 0.5°C / minute rate. The pregnancy diagnosis was performed 30 days after AI. Data were compared by the chi-square test (Excel, Microsoft), with 5% of significance level. A great variation in the progression of the cervical catheter was observed after clamping the fornix. From 23 ewes submitted to deep IA, it was possible to overpass the entire cervix in 9, whereas in the remainder 14, the deep insemination (after the third cervical ring) was always enabled, without semen reflux. The pregnancy rate was 33.3% in superficial cervical AI, and 52.2% in deep cervical AI. Data do not differ with at 5% of significance level, but it was observed a difference at 9% level suggesting a tendency of better performance with deep insemination. News trials are going to be run in order to increase the number of animals and enable more robust conclusions.

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A021 Physiology of Reproduction in Male and Semen Technology

In vitro heat stress model for *Bos taurus taurus* sperm cells

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Keywords: *in vitro* heat stress, SP-TALP, TL-semen,

Bull heat stress increased testicular temperature reducing sperm production, motility and increasing the percentage of abnormal sperm morphology. Establishment of an *in vitro* heat stress model for bull sperm requires medium validation to evaluate cellular function based on sperm motility and mitochondrial activity. SP-TALP (Tyrode-albumin-lactate-piruvate) and TL-semen chemical composition are similar, except for BSA (Bovine Serum Albumin), lactate and piruvate present on SP-TALP (Bavister, Biol Reprod, 16:228-237,1977). Therefore, the objective of this study was to establish an *in vitro* heat stress model for *Bos taurus taurus* sperm evaluating motility and mitochondrial activity. Frozen semen straws (n=30) from four Holstein bulls were used (N= pool of 3 straws/replicate). Samples were evaluated immediately after Pecoll Gradient (0 hour) and after SP-TALP and TL-semen incubation at 35°C, 38.5°C and 41°C during 4 hours. Sperm motility was determined at 0, 1, 2, 3 and 4 hour incubation and mitochondrial activity (Hoechst 33342/MitotrackerRed) at 0 and 4 hour incubation. Data were submitted to ANOVA and non parametric data were analyzed by Wilcoxon using SAS 9.0. SP-TALP sperm incubation at 35°C (54 ± 4.07%); 38.5°C (53 ± 4.07%) and 41°C (47 ± 4.07%) for 2 hours did not affect motility as compared to 0 h (64 ± 4.07%). However, SP-TALP sperm incubation at 41°C for 3 hours reduced (34 ± 4.07; $P < 0.02$) sperm motility as compared to 0 h (64 ± 4.07%). TL-semen sperm incubation at 41°C for 1 h reduced motility (22 ± 3.87%; $P < 0.05$) as compared to 35°C (43 ± 3.87%; $P < 0.05$) and control 0 h (46 ± 3.87%). Incubation of sperm at 38.5°C (1.42 ± 0.017 arbitrary units (AU), $P = 0.05$) and at 41°C (1.41 ± 0,017 UA; $P < 0.05$) for 4 hours reduced mitochondrial activity as compared to 35°C (1.48 ± 0.017 AU; $P < 0.05$) regardless of medium. Mitochondrial activity of sperm incubated in SP-TALP medium (1.44 ± 0.014 AU) was superior ($P < 0.05$) than TL-semen (1.43 ± 0.014 AU) regardless of temperature. These observations suggested that important medium compounds for sperm function are lost in sperm incubated in TL-semen, but it was maintained if incorporated in SP-TALP. In conclusion, *in vitro* heat stress model using SP-TALP medium provided enriched energy substrate for sperm motility and mitochondrial activity.

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A022 Physiology of Reproduction in Male and Semen Technology

The use of cushion solution during sperm *in vitro* selection reduces oxidative stress

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Keywords: cushion fluid, IVP, spermatozoa.

Despite advancements and modifications on the Percoll discontinuous density gradient method for sperm selection in vitro, the centrifugation step in this technique is liable to cause damage to sperm cells. This process may induce the production of reactive oxygen species (ROS) and decrease antioxidant defenses. With the view to minimize the potential damage caused by the centrifugation, a cushioned technique was developed, using a colloid (cushion media) for processing the equine and porcine pre-freezing semen. However, studies using this technique for selecting bovine sperm for IVP are nonexistent. This study aimed to assess the quality of sperm through the process of sperm selection by mini-Percoll gradient modified method (Guimarães et al., Anim Reprod Sci, v.146, p.103-10, 2014), using the cushioned during centrifugation, by analyzing the production of reactive oxygen species and total antioxidant capacity. Six replicates were performed, where straws of a Bos taurus bull were thawed and divided into four treatment groups: Control (C) with only the Percoll discontinuous density gradient; treatment C1, which was added 150μL of cushioned media (CushionFluid® - Minitube, Tiefenbach, Germany) under the gradient in the first centrifugation; C2, with the addition of the same amount of the colloid in the second centrifugation and C1-2 with both cushioned centrifugation. After the selection process, the semen samples were designed for biochemical assays. The ROS levels were determined by a spectrofluorimetric method using 2',7'-dichlorofluorescein diacetate (DCF-DA), with the results expressed in units of fluorescence (UF) (Loetchutinat et al., Radiat Phys Chem, vol. 72, p. 323-31, 2005); the total antioxidant capacity was determined by reducing ferric antioxidant potential (FRAP), using a standard curve of a compound with a known antioxidant activity, and the results are expressed in μg equivalent of ascorbic acid (Benzie and Strain, Anal Biochem, v. 239, p. 70-6, 1996). The data were evaluated by ANOVA and compared with Duncan test ($P < 0.05$). The control group showed increased production of ROS when compared to treatment with Cushion Fluid® (C1, C2 and C1-2) (0.352; 0.270; 0.267 and 0.258 UF, respectively). The antioxidant capacity of the C2 group (44.27) was lower than the treatments C and C1 (58.6 and 58.84) and similar to C1-2 (50.4). These results suggest that sperm selection by Percoll discontinuous density gradient with Cushion Fluid® decreases the ROS levels whereas the sperm total antioxidant capacity was reduced in the group that cushioned method was used in the second centrifugation.

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A023 Physiology of Reproduction in Male and Semen Technology

Use of melatonin and ferulic acid as promoters of cryopreserved equine sperm

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Keywords: antioxidants, oxidative stress, stallion.

Reactive oxygen species can be responsible for causing damage to the membranes of sperm, DNA fragmentation, among other factors influencing fertility especially in cryopreservation. Melatonin (MEL) is a potent antioxidant amphiphilic (hydro and fat soluble), which makes it able to penetrate any cell compartment. The ferulic acid (FA) is a phenolic compound that exhibits a wide range of therapeutic effects against various diseases due to their potent antioxidant effect. This study aimed to evaluate the effect of antioxidants FA and MEL in cryopreservation of equine semen. Five ejaculates from four stallions were used. Among the treatments, we used two concentrations of each antioxidant (MEL 2 mM, MEL 1μM, FA 0.5mM and FA 1.2mM) beyond the control (conventional freezing extender BotuCrio® - Botupharma, Botucatu, Brazil), totaling five treatments. The parameters analyzed were sperm kinetics with the CASA system (SCA program - Sperm Class Analyzer®), sperm morphology by DIC, plasma and acrossomal membrane integrity mitochondrial membrane potential, using fluorescent probes PI, Hoechst 33342, FITC-PSA and JC- 1 and production of reactive oxygen species (ROS) by the sperm with the fluorescent probe CellRox Deep Red®. Comparisons between treatments were performed by generalized linear model (PROC GLM) of SAS (version 9.3) and the differences between them were located with the Duncan test. The probability of $P \leq 0.05$ was considered significant. The results for the motility characteristics were significant differences in some aspects, but no treatment was superior to the control. The evaluation of sperm morphology showed a decrease in major defects in the samples treated with MEL 2 mM, MEL 1μM and FA 1.2mM,. Regarding to membrane integrity, treatment MEL 1μM showed significantly better in percentages of intact cells (intact plasma membrane, intact acrosome and high mitochondrial membrane potential). Cells with oxidative stress not differ between treatments. Based on the analyzes, it is possible to conclude that the treatment MEL 1μM improves sperm membrane integrity in the equine sperm cryopreservation process.

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A024 Physiology of Reproduction in Male and Semen Technology

Vitrification of epididymal sperm from Iberian ibex (*Capra pyrenaica*)

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Keywords: cryopreservation, Iberian ibex, semen vitrification.

Vitrification, a process in which living cells undergo glasslike solidification, is a relatively new cryopreservation method that can successfully preserve the embryos, oocytes, and even the sperm of certain species. For sperm, at least in the species studied so far, kinetic vitrification would appear to be a better alternative. The simplicity of this sperm cryopreservation technique can be useful in field laboratories for wild species because requires less equipment, is much faster, simpler and cost-effective than conventional freezing. The aim of this work was to evaluate comparatively the effectivity of kinetic vitrification and conventional freezing of epididymal sperm from Iberian Ibex (Capra pyrenaica). Testes were obtained from mature ibexes that were legally hunted in the Tejeda and Almijara Game Reserve, in southern Spain (36ºN latitude, Province of Malaga, Spain) during the rutting season (November/December 2013/2014). Epididymal spermatozoa were collected by the retrograde flushing method, using 1 mL of Tris-citric acid-glucose medium (TCG) at ambient temperature (11-13ºC in the field laboratory). Sperm from left epididymis were frozen with TCG-6% egg yolk and 5% glycerol, and sperm from right epididymis were vitrified with TCG-6% egg yolk with 100 mM sucrose. There weren't differences between treatments (frozen-thawed vs vitrified-warmed sperm) for the percentage of motile sperm, percentage of sperm with membrane integrity determined by the hypo-osmotic swelling test, and percentage of sperm with morphological abnormalities (%). However there were significant differences for quality (score 0-4) of motility (2.4±0.2 and 1.4±0.2), percentage of progressive motility (22.7±4.3 and 7.0±1.6%), percentage of intact acrosome (73.8±4.0 and 55.9±2.5%), percentage of viable sperm according to the nigrosin-eosin staining (45.5±4.1 and 29.2±4.1%), percentage of dead sperm with damaged acrosome (5.5±1.0 and 17.3±2.3%) and percentage of live sperm with intact acrosome (45.1±5.5 and 26.5±4.6) respectively. Although better results were found using the conventional freezing method, given the simplicity of sperm vitrification its use under certain field conditions can be recommended for this type of species. Improvement of the technique might, however, provide better post-vitrification outcomes; new vitrifying solutions and additives should be assessed in future work.

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A025E Physiology of Reproduction in Male and Semen Technology

β-defensin 126 and sperm function in cattle

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Keywords: β-defensin 126, cattle, cauda epididymis, sperm migration.

β-defensins are antimicrobial peptides also thought to have a role in sperm function. In cattle, β-defensin 126 (BD126) has been only detected in the male reproductive tract, with preferentially in the epididymis (Narciandi et al., Immunogenetics 63, 641–651, 2011). The macaque ortholog has been shown to enhance the ability of sperm to migrate through cervical mucus (Tollner et al., Hum. Reprod. 23, 2523–2534, 2008). A mutation in the BD126 gene has been linked to subfertility in men, only explained by reduced ability to penetrate through mucus *in vitro* (Tollner et al., Sci. Transl. Med. 3, 92ra6, 2011). The aim of this study was to examine the role of bovine BD126 in sperm function. Western blot (WB) analysis with a BD126 specific monoclonal antibody demonstrated significant BD126 on bovine sperm which previously published methods for macaque sperm failed to remove. WB analysis also revealed that while BD126 is present on sperm and in seminal plasma from intact bulls, it is undetectable in the ejaculate of vasectomised animals, indicating that it does not originate in the accessory glands. Further analysis demonstrated that the peptide is uniquely present in the cauda epididymis and is absent from sperm recovered from other epididymal regions, thus providing a model to study its function. Confocal analysis revealed immunofluorescent labelling of BD126 specific to the tail and acrosomal region in cauda sperm only, suggesting a role in sperm motility. We therefore hypothesized that addition of cauda fluid to corpus sperm would improve motility and ability to penetrate cervical mucus *in vitro*, and that this may be due to the activity of BD126. Testes were collected from adult bulls at an abattoir and sperm from the corpus and cauda epididymis, as well as cauda epididymal fluid (CEF), were recovered. Corpus sperm were incubated for 1 h with CEF in the absence or presence of BD126 antibody (Ab); untreated corpus and cauda sperm were used as controls. A higher number of cauda than corpus sperm migrated through cervical mucus ($P<0.001$) and addition of CEF increased the number of corpus sperm migrating through this matrix ($P<0.05$). The presence of the BD126 Ab failed to abrogate this effect. Analysis of motility using a computer assisted sperm analysis system indicated higher total and progressive motility in caudal sperm when compared with sperm from the cauda ($P<0.001$); again, addition of CEF increased progressive motility ($P<0.05$). In conclusion, we have characterised the expression of bovine BD126 as a protein in the cauda epididymis. Incubation of sperm from the corpus epididymis (which lack BD126) with CEF from the cauda (which contains BD126) resulted in enhanced sperm migration through cervical mucus, and higher motility. Further work will clarify the role of BBD126 and related β-defensins in mediating bovine sperm function.

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A026E Physiology of Reproduction in Male and Semen Technology

Expression of β- nerve growth factor in rabbit male tract and seminal plasma

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Keywords: β-NGF, male reproductive tract, seminal plasma, rabbit

Nerve growth factor (NGF) has been recently identified as an ovulation inductor factor (OIF) in the seminal plasma (SP) (Ratto et al. PNAS 2012; 109:15042-7). The presence of OIF in rabbit has been suggested but this protein has not yet been identified. Our aim was to study the mRNA expression in the rabbit male reproductive tract and to identify the protein β-NGF in the SP. Total RNA was extracted from prostate, testicles and seminal glands of 3 male rabbits (TRIzol® Plus RNA Purification Kit, Life Technologies) to subsequently isolate mRNA (FastTrack® MAG mRNA Isolation Kit, Ambion, Life Technologies,) for retrotranscription to generate cDNA. Specific primers were designed on the mRNA sequence deposited in GenBank (XM_008264614.1) to target a highly conserved region of NGF among species (5'-AGCCCACTGGACTAAACTGCA-3';5'-TCGCACACCGAGAACTCTCC-3'; product size: 305 nucleotides). PCR was performed on cDNA to obtain the expected 300 pb fragment that was sequenced confirming the presence of NGF-mRNA in seminal plasma, testicle and prostate. To determine the expression of mature NGF protein in SP, an aliquot was prepared from collected semen, centrifuged at 3000xg for 30 min at 4ºC and stored at -20°C. For Western blot (WB) analysis, samples were loaded in 12% SDS–PAGE and electrotransferred onto nitrocellulose membranes. The membranes were probed with mouse β-NGF antibody (Promega) using donkey anti-mouse as secondary antibody (Li.Cor Biotechnology). Blots were scanned in an Odissey Infrared imaging system. In addition, NGF was purified by offgel technique with the 3100 OFFGEL Kit pH 3–10 (Agilent Technologies Inc) and the recovered fraction recognized with the mouse β-NGF antibody was used for mass spectrometry analysis (MS) (4800 Plus Proteomics Analyzer Applied Biosystems,). MS was operated in positive reflector mode with an accelerating voltage of 20,000 V. For protein identification NCBInr was used. Database without taxonomy restriction and a home-made database with the sequence of NGF (gi|655847230) downloaded from NCBInr was searched using MASCOT v 2.3. The probability scores of NGF sequences from several species were greater than the score fixed by MASCOT as significant with a p-value < 0.05. Our results show that expression of NGF-mRNA were clearly identified in the rabbit male tract organs above described and the corresponding mature protein band with a mass of ~60 kDa was also identified by WB whereas a ~13 kDa band was detected in the basic fraction (pH=8.24-8.83) obtained when offgel electrophoresis was performed. Furthermore, protein identification by mass spectrometry revealed the existence of NGF in the SP. In conclusion, mRNA and protein NGF are present in rabbit male reproductive tissue and SP respectively, providing the basis to undertake further functional analysis for its potential role in rabbit reproduction.

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A027E Physiology of Reproduction in Male and Semen Technology

Assessment of bull semen quality loaded in new SensiTemp straws using semen and IVP technologies

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Keywords: bull semen, fertilization, IVP, SensiTemp straws.

SensiTemp, a new IMV bull straw concept, presents the advantage of color changing while the straw is thawed. The color of frozen straws is blue and straws start to become white when the temperature reaches 33°C, with a complete change of color at 37°C. The objective of this study is to assess quality after thawing of semen frozen in SensiTemp, *in vitro*, using Computer Assisted Semen Analysis (CASA), Flow Cytometry (FC) and *In Vitro* Fertilization (IVF). The ejaculates of two bulls, selected during preliminary experiments on high *in vitro* fertility, were harvested at CIA L'Aigle, France and split ejaculates were frozen in experimental (SensiTemp) and conventional (Control) straws. In experiment 1 after thawing semen from the two type of straws (*5 pooled straws each; 2 replicates*), motility was assessed using the IVOS CASA system (Hamilton Thorne Inc., Beverly, MA, USA) and membrane integrity was evaluated through FC with Cytosoft software (Millipore-Guava Technologies Inc., Hayward, CA). In experiment 2, IVF was used to evaluate the non toxicity of SensiTemp and control straws. Cumulus-oocyte complexes (COC; *n=1178; 4 replicates*) collected from slaughterhouse ovaries were matured in IVM medium (TCM-199 with bicarbonate, Sigma-Aldrich, Saint Quentin Fallavier, France; 10µg/ml FSH-LH, Reprobiol, Liège, Belgium and 10% FCS, Thermo Fisher, Illkirch, France) for 22 h. After fertilization, presumptive zygotes of each group (SensiTemp and control for each bull) were cultured in synthetic oviduct fluid medium (SOF, Minitube, Tiefenbach, Germany) with 1% ECS and 0.6% BSA (Sigma-Aldrich, France) up to 8 days. All cultures were conducted at 38.5C in 5%CO2, 5%O2. The cleavage and blastocysts rates were evaluated on Day 3 and 7, respectively for each group. Embryo quality was recorded on day 7 according to the IETS evaluation. Data from each bull were analyzed separately using the Chi square test ($P<0.05$). In experiment 1, neither sperm motility from bull 1 (61.2 and 60.5%) and bull 2 (66.2 and 66.5%) nor membrane integrity from bull 1 (58.6 and 52.2%) and bull 2 (61.0 and 61.9%) were different between SensiTemp and Control, respectively. Results from experiment 2 showed no difference ($P>0.05$) in cleavage rate between SensiTemp and Control for the two bulls: 92.1 and 91.7% for bull 1 and 94.2 and 94.6% for bull 2 respectively. The blastocysts rate on day 7 did not differ ($P>0.05$) among groups (47.5, 47.1 and 51.3, 50.4% for SensiTemp and Control bull 1 and bull 2, respectively) nor the quality of embryos retrieved in the different groups: 25.4, 23.3 and 30.8, 29.6% in grade 1 embryo for SensiTemp and Control bull 1 and bull 2, respectively. Those results demonstrate, *in vitro*, that the new SensiTemp straws were non toxic and did not affect the semen quality after thawing nor did the SensiTemp straws affect the ability of sperm cells to fertilize oocytes and produce 8 days old embryos.

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A028E Physiology of Reproduction in Male and Semen Technology

Hyaluronic acid-binding ability of spermatozoa and its role for selection of vacuole free human spermatozoa in human reproduction

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Keywords: ART outcomes, DNA integrity, sperm head vacuoles, MSOME, sperm selection, hyaluronic acid binding.

The type of spermatozoa selected in ART (assisted reproductive technology) influences the outcome in regard to embryo development, pregnancy, miscarriage and malformation. Sperm head nuclear abnormalities were identified earlier as vacuoles by motile-sperm organelle-morphology examination (MSOME). Blastocyst development and the pregnancy rates are negatively influenced if vacuoles containing sperm are used for ICSI. Thus, it is of importance to reliably select vacuole-free spermatozoa in assisted reproduction. In a prospective, observer blinded study. Hyaluronic acid (HA) bound, standard morphological (SM) selected (200x) and unselected sperm were collected by different examiners. The evaluation of vacuoles by Nomarski differential interference contrast (DIC; 600x up to 7.200x) was performed observer blinded for all samples. Eleven human semen samples were prepared by a 80% density gradient. From each sample a minimum of 20 sperm per method (HA and SM selection) were collected in separate PVP droplets. Additionally, 20 unselected spermatozoa were collected from each sample designated as control. The number of vacuoles in each sperm head was determined by means of DIC. One way analysis of variance was performed (Tukey-Test; Sigma Stat Version 3.5, DUNDAS Software LTD.). Significantly more sperm without vacuoles were found in HA selected ($p<0.001$) and SM selected ($p<0.001$) than in unselected samples. The number of sperm with one or two vacuoles ($p<0.01$) and more than two vacuoles ($p<0.001$) was significantly higher in the unselected group. Furthermore, in HA selected sperm the appearance of two vacuoles was significant lower than in SM selected sperm ($p<0.05$). Both selection methods provide spermatozoa containing less vacuoles than in the unselected samples, especially in the group with more than two vacuoles. This shows that HA selection is a good method to select spermatozoa in regard to the appearance of vacuoles. This is of significance since the HA selected spermatozoa are more mature, with less cytoplasmatic retention and higher DNA integrity than unselected sperm cells. Thus HA selection may be an effective method to identify spermatozoa with a higher potential in reproduction in order to improve safety and results in ART procedures.

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A029E Physiology of Reproduction in Male and Semen Technology

Seasonal variation of testicular functionality in alpaca (*Vicugna pacos*) raised in Italy

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Keywords: alpaca, scrotal edema, testicular measures, ultrasonography.

Thermoregulatory functions of testicles are very important for sperm viability in terms of spermatogenesis and maturation phases. Unique characteristic are present in South American camelids related to the position, body mass/testicle volume ratio and anatomical features (epididymis orientation). Among different parameters to evaluate male there are testicular dimension measures (width, length and thickness). It has been observed different times the increase of testicular volume during heat season in animals with pendulous testicles because of circulatory impairment. Aim of this study was to monitoring physical and physics parameters besides the semen quality evaluation during two different seasons (summer, winter) in alpacas. Eight adult males are evaluated considering classical (testicular measures – caliper measurements) and innovative parameters as ultrasonography of the testicles. Semen collections were performed with a teaser and ejaculates obtained were destined to the quality assessment (volume, colour, viscosity, motility and concentration) and biochemical evaluation of the seminal plasma (energetic, protein and enzymatic profile – Hitachi 912 biochemical auto-analyzer). Data were analysed for ONE-WAY ANOVA considering the season as variable independent and the parameters evaluated as dependent variables using the statistical software SIGMASTAT 2.05. There was a significant difference among seasons with a general decrease of the semen quality during the hot season. The lower levels of volume, concentration, seminal plasma (SP) glucose, SP cholesterol, SP triglyceride, SP Phosphates and the higher levels of SP Gamma Glutamil Transferase, SP Alkaline Phosphatase, SP Magnesiumn clearly indicate a detrimental effect of high environmental temperature because the effect on testicular thermoregulatory capability. Negative correlation between Testicular Measures and semen quality parameters was significant (r: -0.64 - -0.45) . At the ultrasound evaluation was characterized the reason of increased testicular mass during the hot season considering the evidence of scrotal edema. The scrotal edema derived by a defect of local circulatory mechanism. Testicular functionality may be influenced by the high environmental temperature and specifically in alpaca were the position of the gonads imposes a fine regulatory pattern. Hot season causes a testicular circulatory defect with a scrotal edema as results and a decrease of semen quality.

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A030E Physiology of Reproduction in Male and Semen Technology

Characterization of accessory glands ultrasonography in rams of endangered venetian sheep breeds

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Keywords: accessory glands, endangered Venetian Sheep Breeds, breeding soundness evaluation, ultrasonography.

Evaluation of male needs standardized protocol for the male's classification. Breeding soundness evaluation (BSE) is a practice that is widely used, mainly in the bull, to evaluate the male starting from physical and reproductive parameters. BSE protocols for rams are already published around the world but complete evaluation is not yet raised for all the breeds with specific characteristics. The male can be classified as Satisfactory, Questionable and Unsatisfactory. When is not possible to evaluate it properly, the classification can be deferred. Among the innovative methods to perform the evaluation, there is the ultrasound exam of the reproductive organs. Testicles, epididymis, vascular cone and accessory glands ultrasound may increase the accuracy of the evaluation. In this study, an established sample of rams belonging to Veneto Agricoltura Center in Villiago (BL), Italy, has been evaluated with classical and innovative monitoring system. On these animals the entire BSE procedure was carried out. Moreover, the ultrasound evaluation (MyLabVet^{TM} One, ESAOTE S.p.A., Genova, 10 MhZ probe frequency) of testicles and vesicular glands has been performed for the first time in these breeds (18 adult rams: N=5 Brogna, N=5 Lamon, N=4 Foza, N=4 Alpagota). After the physical and physics exams all the males involved in the evaluation were collected using electro-ejaculator (Ruakura Ram Probe Plastic Products, Hamilton, New Zeland); the trans-rectal probe was inserted after a mucosal anesthesia (5 ml of Lidocaine 2 %) performed during the deferent ampullas massage. Procedure of semen quality evaluation considering general ejaculate parameters (color, volume, concentration) and specific microscopic observation about viability fresh-post thawed with differential staining (Eosin/Nigrosin, Spermac and Farrelly staining), kinetic CASA parameters (Ivos II, Hamilton Thorne, Germany). Data analysis (Pearson correlation indices) revealed important correlations among scrotal circumference, serum testosterone and semen kinetic parameters. Furthermore, increasing the testicular parenchyma echogenicity, the semen volume used to lower. Testicular and vesicular glands ultrasound exam give us important information about seminal plasma quantity. Particularly vesicular glands echogenicity has shown high relationship with quantity of seminal plasma and therefore low sperm concentration. Physics equipment as ultrasonography may optimize collection procedure performed with electro-ejaculator. Body mass and vesicular glands dimension can influence the induction success and the semen freezeability.

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A031E Physiology of Reproduction in Male and Semen Technology

The joint treatment of sperm by prolactine and GTP have determined the increase of the number acrosome-reacted spermatozoa in bulls

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Keywords: acrosoma-reacted spermatozoa, bulls, prolactin.

There are contradictory opinions concerning involvement of prolactin (PRL) in the process of sperm capacitation and acrosome reaction (Vigil P. et al., 2011 Biol Res, 44:151-159). It was shown that PRL stimulates release of Ca^{2+} from IP3-sensitive stores, and GTP stimulates release of this ion from IP3-insensitive stores (Denisenko V. et al., 2015 Tsitologiya, 3:1-8). GTP forms a connection between IP3-sensitive and IP3-insensitive intracellular stores and promotes transition of Ca^{2+} between these stores (Mullaney J. et al., 1987 J. Biol. Chem. 262: 13865—13872). The aim of the present study was to examine the mobilization of Ca^{2+} from intracellular stores after the treatment spermatozoa by PRL and GTP and to evaluate the status of spermatozoa after these treatments. Intensity of fluorescence of membrane-bound Ca^{2+} was determined with a fluorescence spectrophotometer Hitachi MPF-4 (excitation: 380-400nm, emission: 530 nm) using 40 μM chlortetracycline (CTC) - (Denisenko V. et al., Tsitologiya 3:1-8, 2015). Intensity of fluorescence of membrane-bound Ca^{2+} was determined in Sp-TALP medium where the concentration of cells was adjusted to 1, 5 X 10^6 sperm/mL. The CTC assay was used to determine the functional status of spermatozoa (Ded L. et al., 2010 Reprod Biol Endocrinol, 8-87). Samples were examined with fluorescence microscope *Zeiss Axo Imager* M1. Ejaculates from three fertile bulls were used, and five replicates were performed for each experiment. In each sample, 200 cells were evaluated. Sperm were evaluated according to 1 of 3 CTC staining patterns: fluorescence over the entire head (precapacitated cells), fluorescence-free band in the postacrosomal region (capacitated cells) and low fluorescence over the entire head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells). All reagents that were used in this study were produced by Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Treatment spermatozoa by PRL (10 ng/ml) or GTP (10 μmol) resulted in release of Ca^{2+} from intracellular stores (0.70±0.019 and 0.69±0.017 vs 0.85±0.016; $P<0.001$). There was additional release of Ca^{2+} with the combined effect of PRL and GTP (0.62±0.011 *vs* 0.70±0.019 and 0.69±0.017; $P<0.001$). There was no additional release of Ca^{2+} after the joint action by the pair of these reagents in the presence of protein kinase C inhibitor (Ro 31-8220, 10ng/ml). The average percentages of capacitated spermatozoa did not change after treatment by PRL, GTP or both these reagents. The percentage of cells that underwent acrosome reaction have increased after treatment by PRL and GTP jointly (46% *vs* 62%, $P<0.01$); there was no such effect at preliminary treatment of sperm by Ro 31-8220 (10 ng/ml). Thus, Ca^{2+} transition between intracellular stores in bull spermatozoa after the treatment with PRL and GTP jointly is leading to increasing in the percentage of acrosome-reacted spermatozoa.

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A032 Folliculogenesis, Oogenesis and Ovulation

PI3K-Akt signaling pathway association with oocyte competence as indicated by mirna profiling and quality assessment in cattle

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Keywords: microRNA, oocyte competence, PTEN and Akt.

In mammals, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes, and therefore, regulate many signaling pathways and cellular process. We hypothesized that intracellular pathways highly regulated by miRNAs in follicular cells are involved in acquisition of oocyte competence. To test this hypothesis slaughterhouse ovaries (3-6mm) were dissected and ruptured for recovery of granulosa cells (GC) and cumulus-oocyte complex (COC), these cells were counted and placed in culture for 96 hours and then used to RNA extraction. A total of 351 miRNAs were profiled in GC and COC in order to identify miRNA-regulated pathways acting into the follicular microenvironment. Were identified 305 miRNAs present in both cell types, GCs and COCs, 8 unique in GCs and 13 exclusive in COCs. Bioinformatic analysis of unique miRNAs for each cell type showed 69 and 85 pathways, respectively, are predicted to be miRNAs regulated. One of the identified pathways, both in GC and COC, was the PI3K-Akt signaling pathway. To test its association with oocyte competence we dissected bovine ovarian follicles ranging from 3-6mm and recovered follicular cells (GC and cumulus cells) and oocytes; the former was stored and the latter were assigned for individually in vitro matured (IVM), parthenogenetically activated and in vitro cultured (IVC) until the blastocyst stage. Individual IVM, activation and IVC were performed in order to track follicular cells, obtained after dissection, with the oocyte fate after activation as follows: non-cleaved oocytes (Ncleav group), mature oocytes that cleaved and did not reach blastocyst stage (Cleav group) or that reached blastocyst stage (Blast group). Follicular cells from these three groups were used to search for the following PI3K-Akt pathway components: phosphorylated-Akt (p-Akt) protein levels (a positive regulator of PI3K-Akt pathway) and PTEN mRNA levels (a negative regulator of PI3K-Akt pathway. Determination of p-Akt levels indicated higher ($p=0.06$) levels of Akt in the Blast group (0.74 ± 0.33) compared to Cleav group (0.45 ± 0.39), while both were equal to Ncleav group (0.59 ± 0.43). PTEN expression in follicular cells from the Blast group (0.60 ± 0.10) was lower ($p<0.05$) than Cleav (2.42 ± 1.55) and Ncleav (2.23 ± 0.47) groups. Taken together these results demonstrated that the miRNA expression profile in follicular cells consists in a useful tool to identify putative molecular pathways involved in oocyte competence acquisition. This principle was given proof by the determination of p-Akt and PTEN levels in follicular cells from ovarian follicles carrying oocytes with distinct developmental competence, which indicate the association of PI3K-Akt signaling pathway activation with oocyte competence.

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A033 Folliculogenesis, Oogenesis and Ovulation

Evaluation of glucose and lactate production by canine luteal cells in early cyclic and gestational diestrus

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Keywords: canine, glucose, luteal.

There is great similarity in several aspects between the cyclic and pregnant canine luteal phase. In order to investigate the metabolic pathways of luteal cells in early cyclic and gestational diestrus, we aimed to analyze glucose and lactate production by these cells cultured in vitro. Ovariohysterectomy was performed in 6 females, 3 in early gestational diestrus and 3 in initial cyclic diestrus. Corpora lutea (CL) were enzymatically digested in a solution containing 5 ml DMEM (Dulbecco's Modified Eagle's Medium - high glucose, Sigma -Aldrich, Germany) and 0.0075g collagenase type 1 (Collagenase from Clostridium histolyticum Type I, Sigma -Aldrich, Germany). After one hour at 37.5°C and vortexing every 15 minutes, the cell suspension was filtered (Filter with 70 µm, BD Falcon®, USA), centrifuged 3 times (340G, 220G and 100G respectively at 20°C for 10 min) and resuspended in 12 ml DMEM supplemented with antibiotic, antifungal, L-glutamine (Sigma -Aldrich, USA) and fetal serum bovine (Sigma -Aldrich, EUA). The solution containing luteal cells (experimental solution) was distributed in 24 well plates and incubated in controlled atmosphere (containing 5% CO2 and 95% air). After 24 hours, wells were washed with HBSS (14,175,079; Gibco BRL) and medium replaced. A plate containing only culture medium was used as control (control solution). Experimental and control solution samples were collected 36 (moment 1), 48 (moment 2) and 60 (moment 3) hours after the start of culture, stored in plastic tubes and kept in a freezer at -80°C. Glucose and lactate levels were assessed using VITROS Chemistry Products Calibrator kits (Products Vitro Chemistry, United Kingdom). The statistical analysis was performed using ANOVA ($p < 0.05$) in SAS PROC GLM. We observed that glucose consumption and lactate production increased during in vitro culture in both gestational luteal cells and cyclic diestrus, but glucose consumption and lactate production in gestational CL was greater than cyclic CL at initial (glucose in moment 1, $p=0.0328$ and lactate in moment 2, $p=0.0221$) and final (glucose and lactate in moment 3, $p=0.0085$ and $p=0.0009$, respectively) culture stages. According with these results, we believe that there is no difference in the metabolic pathways used by pregnant and cyclic luteal cells. However, at initial diestrus, energy metabolism appears to be greater in pregnant than cyclic CL.

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A034 Folliculogenesis, Oogenesis and Ovulation

Evaluation of estrous cycle of rats submitted to physical activity and growth hormone

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Keywords: growth hormone, physical exercise, vaginal cytology.

Growth hormone (GH) has been used indiscriminately in order to increase muscle mass. Considering that GH can act on different cell types, including reproductive organs, the aim of this study was to assess the effect of GH treatment, with or without physical activity, on estrous cycles of female rats. Forty female rats aging 9 weeks were sorted into 4 groups: CT (control); Ex (submitted to resisted training); GH (treated with GH); and ExGH (submitted to resisted training and GH treatment). Treated animals received 0.2 IU/Kg of GH every two days, while the others received saline solution at the same volume. Resisted training was performed 3 times a week, and consisted in four series of 10 jumps in the water with 1 minute interval between them. Animals had a vest with 50% of their weigh during the exercise. All rats were submitted to vaginal cytology analysis for 7 days before the experiment to confirm cyclicity. Vaginal cytology was performed daily in all rats for 29 days. Vaginal smears were stained with Panótico® and observed in a microscope. Data normality was tested using the Shapiro-Wilk test, and statistical analysis was performed by ANOVA followed by Tukey's test ($p < 0.05$). No statistical differences were observed between experimental groups for the duration of the luteal and follicular phases of the estrous cycle. However, more cycles were observed in the CT group (7.10 ± 0.88) compared to the other groups: GH (6.10 ± 0.99, $p = 0.04$); Ex (6.10 ± 0.57, $p=0.02$) and ExGH (6.00 ± 0.67, $p = 0.01$). Therefore, it was concluded that GH treatment and resisted training decrease the frequency of estrous cycles during a 29 days period.

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A035 Folliculogenesis, Oogenesis and Ovulation

Ultrasound evaluation of ovarian dynamics in Indubrasil cows submitted to two nutritional managements

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Keywords: estrous cycle, feeding, reproduction.

The objective of this study was to compare ovarian dynamics of Indubrasil cows submitted to grazing or confinement regimes. Follicular growth was monitored daily starting at estrus during two consecutive estrous cycles in seven adult non-lactating Indubrasil cows with body score 4 (classification 1 to 5), in 2x7 experiment. The first evaluation was in the grazing regime, when animals were kept in pastures of *Brachiaria brizantha* cv Marandu intercropped with *Leucaena leococephala*, with water and mineral salt ad libitum. The second evaluation was performed during the following estrous cycle when animals were submitted to the confinement regime, and fed with corn silage, proteinated mineral salt and water ad libitum. An ultrasound HS 1500 (Honda®, Japan) equipped with a linear 7.5 MHZ probe was used to assess follicle and luteal dynamics. Data were recorded according to the day of the cycle and are presented as means ± standard deviation. Means were compared by T Tests and differences considered significant when $P<0.05$. The duration of the first estrous was 20±1.6 and 21±1.22 days for grazing and confined animals, respectively. The number of follicular waves was 2±0.49 and 3±0.40 and their duration was 9±1.99 and 9±1.65 days, for grazing and confined animals, respectively. The maximum diameter of the ovulatory follicle was larger in confined (15±0.51mm; $p <0.05$) than grazing animals (13±0.87mm). For subordinate follicles, the maximum diameter was 9±1.13mm and 9±0.82mm for grazing and confined animals, respectively. The dominant follicle persisted for 7±1.41 and 6±0.82 days in grazing and confined animals, respectively. Finally, the persistence of the corpus luteum was 14±1.59 and 15±1.06 days for grazing and confined animals, respectively. These data suggest that diet influences growth rate and size of the ovulatory follicle in Indubrasil cows. We speculate that greater dry matter intake in the confinement regime may have promoted an increase in the metabolism of steroid hormones, influencing dominant follicle growth. This abstract describes for the first time follicle dynamics in Indubrasil cows, which was found to be very similar to other *Bos indicus* breeds.

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A036 Folliculogenesis, Oogenesis and Ovulation

Characterization of ovarian follicle reserve depletion in Ames dwarf mice

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Keywords: aging, follicles, ovarian reserve.

Ames dwarf mice are deficient in growth hormone (GH) secretion and therefore have reduced levels of insulin-like growth factor type I (IGF-I). Their life expectancy is around 30-50% longer than normal mice (Masoro; Mech Ageing Dev; 2005; 126: 913-22). Studies have shown that the IGF-I signaling pathway is involved in the regulation of follicular growth and development (Ahmed & Farquharson; J Endocrinol, 2010; 206: 249-59). Therefore, the aim of this study was to evaluate the number of follicles in the primordial, primary, secondary and tertiary stages, as well as follicular and oocyte diameters in Ames Dwarf and Normal mice. Ovaries of normal (n=3) and Ames Dwarf (n=4) mice at 12 months of age were used. Ovarian samples were processed, sequentially cut and stained with hematoxylin-eosin. Ovarian sections were assessed in an optical microscope using 10x and 40x objectives for the classification, quantification and measurement of ovarian structures. One of each six histological sections per slide was evaluated. Statistical analyzes was performed using t-tests with the GraphPad Prism 5 software (La Jolla, CA, USA). Differences were considered significant when $P<0.05$. Ames dwarf mice presented ($P=0.001$) more primordial follicles (1548 ± 139) compared to normal mice (378 ± 125). These data indicate that primordial follicles are not progressing to the primary stage, possibly due to reduced serum levels of IGF-I and insulin, indicating that these mice can be a good model to study the relationship between metabolic status and ovarian aging. The number of secondary and tertiary follicles was not different between Ames dwarf and normal mice ($P>0.05$). The total number of follicles tended ($P=0.07$) to be higher in Ames dwarf (2,673 ± 209) than in normal mice (1,668 ± 445). Regarding follicle and oocyte diameters, we only observed a larger diameter ($P=0.02$) for oocytes included in primordial follicles from normal (6.8 ± 0.4 µm) compared to Ames Dwarf mice (5.5 ± 0.3 µm). In conclusion, Ames Dwarf mice have more primordial follicles compared to normal mice, suggesting that GH, IGF-I and insulin deficiency leads to the accumulation of follicles in the primordial stage and increase ovarian longevity.

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A037 Folliculogenesis, Oogenesis and Ovulation

Green tea influence on VEGF expression in the rat ovary

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Keywords: *Camellia sinensis*, corpus luteum, reproduction.

Green tea is derived from *Camellia sinensis* and considered a healthy drink as it is associated with prevention of cancer, cardiovascular disease and osteoporosis. It contains catechins, which appear to play antioxidative, antibacterial and antitumoral roles. Despite many potential benefits of green tea consumption, the catechins present in green tea can significantly inhibit proliferation, steroidogenesis and vascular endothelial growth factor (VEGF) production by swine granullosa cells. Therefore, it is important to assess possible reproductive-related consequences. The aim of this work was to assess green tea influence on VEGF expression in the rat corpus luteum. Wistar rats were divided in two groups: the control group (n=30) had access to water ad libitum, and in the treated group (n=30) water was replaced by a commercial green tea (Amor à Vida®, Amor à Vida Produtos Naturais, Brazil) at 2.5% ad libitum as previously performed by Yang et al. (Eur J Cancer Prev, 12:391–395, 2003) and Niwattisaiwong et al. (Drug Metabol Drug Interact., 20:43-56, 2004). Rats were kept in plastic boxes (5 animals per box) with free access to beverage and food. This study was approved by the local ethics committee. The experiment lasted for three months and at the end of each month 10 animals of each group were superovulated with 150IU/Kg of eCG (Folligon®, Intervet Schering-Plough, Brazil) and 150IU/Kg of hCG (Vetecor®, Hertape Calier, Brazil) and killed. Messenger RNA was isolated from corpora lutea with TRIzol® (Life Technologies, Brazil) and reverse transcription was performed with SuperScript III RT Kit (Life Technologies, Brazil) and Oligo (dT) primers (Life Technologies, Brazil). Abundance of VEGF mRNA was assessed by qPCR using TaqMan Ral-Time PCR Master Mix (Life Technologies, Brazil) and HPRT-1 as the housekeeping gene. Relative VEGF mRNA abundance was calculated using the ΔΔCT method and Pfaffl's method for efficiency correction (Nucleic Acids Research, 29:2004-2007, 2001). Statistical analysis was performed with unpaired t tests and the moments for each group were compared by analysis of variance. Differences were considered significant when $p<0.05$. Green tea consumption per rat per day was greater (30.73±0.49) than water consumption (29.13±0.50). No differences were observed between groups for VEGF expression. Mean values and standard errors for VEGF mRNA abundance were: first month (control = 1.07±0.13 and treated = 1.05±0.14); second month (control = 1.09±0.14 and treated = 0.94±0.11); and third month (control = 1.05±0.11 and treated = 0.73±0.10). In conclusion, consumption of green tea for three months did not alter abundance of VEGF mRNA in the rat corpus luteum.

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A038 Folliculogenesis, Oogenesis and Ovulation

Estradiol concentration and gene expression of mRNA for CYP19A1, PAPP-A and LHR in dominant and subordinate follicles at follicle deviation in Nelore cows

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Keywords: bovine, follicle selection, gene.

The main objective of this experiment was to investigate expression of mRNA of genes associated with follicle deviation, *CYP19A1* (aromatase), LH receptor (*LHR*) and *PAPPA* (Pappalysin 1), using an *in vivo* model to obtain granulosa cells. Nelore (NEL; n = 10), non-lactating, 3-6 years old females, had follicle wave emergence synchronized by transvaginal aspiration of follicles ≥5 mm in a cross-over design. At the same time, they received an intravaginal progesterone device (CIDR®, Zoetis, Brazil) and 24h later, two doses with 0.5 mg of cloprostenol with 12h interval, with luteolysis monitored by ultrasound. Ovaries were evaluated every 12h to characterize follicular dynamics. A model based on follicular aspiration was used, and cows were distributed among three treatments: group 0h, when the largest follicle reached 6.5 mm, the two largest follicles were aspirated (DF0h and SF0h); group 12h, the two largest follicles (DF12h and SF12h) were aspirated 12h after the largest follicle reached 6.5 mm; and deviation group, the largest follicle (DF0h) was aspirated when it reached 6.5 mm, and the second largest follicle (SF→DF) was aspirated 12h later. Granulosa cells were obtained by washing the follicle cavity by successive aspiration and ejection with 1 mL of sterile saline solution, using an aspiration system with double lumen. The suspension was centrifuged and the supernatant was stored. Follicular fluid estradiol-17β (E2) concentration was measured by ELISA. The cell pellet was suspended in lysis buffer from RNeasy kit (Qiagen, SP, Brazil) and mRNA expression was analyzed by RT-PCR for *LHR*, *CYP19A1* and *PAPPA*. Statistical analysis for follicular dynamics data and gene expression was performed using the PROC MIXED of SAS. Samples contaminated with blood or E2 concentration below 1 ng/mL were removed from the analysis. Follicular fluid E2 concentration (ng/mL) from SF→DF (176.9 ± 48.8; n = 4) was higher (P≤0.02) than from SF0h (34.2 ± 35.8; n = 7) and SF12h (28.9 ± 46.0; n = 4), and did not differ (P>0.05) from DF0h (188.9 ± 30.3; n = 7) and DF12h (244.4 ± 46.0; n = 4). There was no difference (P>0.05) among groups in relative mRNA expression for *PAPPA* (DF0h: 0.14 ± 0.05; SF0h: 0.14 ± 0.07; SF→DF: 0.31 ± 0.08; DF12h: 0.14 ± 0.08, and SF12h: 0.14 ± 0.08) and *CYP19A1* (DF0h: 0.38 ± 0.13; SF0h: 0.27 ± 0.15; SF→DF: 0.49 ± 0.20; DF12h: 0.47 ± 0.19, and SF12h: 0.06 ± 0.19). There was a tendency for greater *LHR* mRNA expression in SF→DF follicles (3.86 ± 1.43) compared to group 0h (DF0h: 0.56 ± 0.86, P=0.07, and SF0h: 0.46 ± 1.08; P=0.06). There were no differences in gene expression between SF→DF and the 12h group (DF12h: 5.71 ± 1.43 and SF12h: 1.04 ± 1.43). DF12h *LHR* expression was highest in groups 0h (DF0h and SF0h; P=0.01) and SF12h (P=0.05). We concluded that increased expression of the LH receptor was the main early mark of dominance in Nelore cows.

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A039 Folliculogenesis, Oogenesis and Ovulation

Extracellular vesicles contents isolated from bovine ovarian follicles: association with oocyte competence

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Keywords: bovine, exosomes, follicular fluid.

Intercellular communication is crucial to induce cell proliferation and differentiation during follicle growth. One consequence of follicular growth is production of a viable oocyte capable to generate a pregnancy. Part of the crosstalk occurs in the follicular fluid (FF). Extracellular vesicles (EVs) such as exosomes and microvesicles were identified within the follicular fluid and can mediate cell communication. Extracellular-vesicles can transfer bioactive molecules such as lipids, proteins, mRNA and miRNAs. Our hypothesis is that exosomes from bovine follicular fluid present coding RNA molecules and different lipid composition associated with oocyte competence. In order to test this hypothesis bovine ovarian follicles, between 3-6 mm were individually isolated. Follicle contents were separated under a stereomicroscope to allow the collection of FF and the cumulus-oocyte-complex (COC). Follicular fluid was submitted to differential centrifugation for removal of cellular components and debris before freezing at -80°C. COCs were in vitro matured for 18h and then evaluated for the presence of the 1st polar body. Activation of the denuded oocytes to generate parthenogenetic embryos started 26h after the beginning of maturation. After seven days in culture we accessed the oocyte competence and grouped the follicular fluid according to developmental competence. Developing groups were based on the ability or inability of the oocytes to mature and generate a blastocyst. Based on the competence groups we isolated EVs from pools of 10 follicular fluids. Total RNA and lipids were extracted from the pools of EVs and analyzed by Next Generation Sequencing and tandem mass spectrometry. Transcriptome analysis demonstrated the presence of coding RNA species including HDAC2, HDAC10, EIF4E, EIF2B e BRCA1. Functional annotation analysis of exosomal RNA content demonstrated to be enriched for RNA molecules involved in the regulation of chromatin remodeling or transcription activation. Based on lipids analysis we identified different lipids enriched according to the development competence of the oocytes. We identified six lipids associated with poor oocyte competence; monoalkenyl diacylglycerol (MADAG 52:8+NH4 (-FA 18:1 (NH4) and MADAG 48:8+NH4 (-FA 16:1 (NH4) are examples of the lipids found in extracellular vesicles. Three lipids were associated with oocyte capability to generate a blastocyst, for example Digalactosyldiacylglycerol (DGDG 36:2+NH4 (-DGDG (NH4)), which was identified present in extracellular vesicles. Thus, our results demonstrated that EVs carry coding RNA molecules involved in chromatin regulation. Also, lipids identified are differently expressed in EVs and are associated with oocyte competence. Further experiments are necessary to explore the different lipid molecules present in the EVs and their role during follicle growth and oocyte maturation.

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A040 Folliculogenesis, Oogenesis and Ovulation

Correlation between phenotype, genotype and antral follicle population in *indicus-taurus* heifers

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Keywords: antral follicles count, genetic improvement, heifers.

The objective of this study was to assess the potential correlation between phenotypic and genotypic characteristics with antral follicle count (AFC) in *indicus-taurus* heifers. Braford heifers (Nelore 3/8 x 5/8 Hereford, 18-24 months n = 270) from an genetic improvement program, Conexão Delta G, were evaluated by transvaginal ultrasonography micro convex transducer (7.5 MHz) on a random day of the estrous cycle to determine AFC (follicles > 3 mm diameter), according to Burns et al. Biology of Reproduction (2005). After AFC evaluations (average of 26.81 follicles), the number of antral follicles was correlated with genetic selection parameters using 4 statistical models. In model 1 (n = 270) we consider the effect of contemporary group (CG) and the covariates: age (AG); weight gain from birth to weaning (GW); visual scores for conformation at weaning (CW), precocity at weaning (PW) and musculature at weaning (MW). In Model 2 (n = 270) we considered the effect of CG and covariates: AG; weight gain from weaning to yearling (GY); visual scores for conformation at yearling (GY), precocity at yearling (PY) and musculature at yearling (MY). The effect, variables and covariates of model 1 and 2 were combined to form model 3 (n = 270). Model 4 (n = 270) consisted of the same parameters of model 3 with the inclusion of the paternal effect (sire). Data were analyzed by linear regression using the GLM procedure of SAS and adopting $P < 0.05$. From four models tested, the variables and covariates at yearling (model 2) had no effect on AFC ($P > 0.05$) and the coefficient of determination (R2) was 0.056. The other models also showed low correlation with AFC: 0.072, 0.082 and 0.172 for models 1, 3 and 4, respectively. The model with paternal effect was the correlation with the highest score considering genotypic and phenotypic characteristics and AFC. Models 1, 3 and 4 also showed that AFC of indicus-taurus heifers can be influenced by precocity at weaning ($P < 0.05$). Based on this study, there is no correlation between phenotypic and genotypic characteristics with the antral follicle population from *Bos indicus-taurus* heifers. However, AFC can be affected by precocity at weaning. Additionally, we encourage the use of AFC for in vitro embryo production, since there is a quantitative benefit on the number of embryos produced. However, we highlight the genetic merit as the most important criteria for all reproductive techniques.

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A041 Folliculogenesis, Oogenesis and Ovulation

Regional distribution of preantral follicles in equine ovaries

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Keywords: ovary, preantral follicles, region.

Isolation and in vitro culture of preantral follicles in equine ovaries is an emerging technique with several challenges. In addition to the shortage of slaughterhouses for this species, the occurrence of ovarian fragments without preantral follicles is very common. The objective of this study was to define specific ovarian regions in order to increase the probability of a high concentration of follicles from harvested fragments. Ovaries (n=3) of mares in seasonal anestrus were obtained from a local slaughterhouse and transported to the laboratory. Each ovary was divided into two hemiovaries (longitudinal section). Each hemiovary was then sectioned in three parts, each covering the entire length, with the first cut in the region of the small curvature of ovary (C1; near ovulation fossa); the second in the intermediate region between the smaller and larger curvatures (C2; parenchymal region) and the third cut contemplating the greater curvature of the ovary (C3; external region). After that, fragments were immediately fixed in Bouin, kept in 70% ethanol and then processed for histology. A total of 900 slides was prepared with 2.700 histological sections 5 micrometers thick. All preantral follicles containing one oocyte were counted regardless the stage of development (primary, secondary or tertiary) and morphological integrity (normal or degenerated), making the total of 1,514 follicles. Data were submitted to simple logistic regression test ($P \leq 0.05$). All regions showed differences ($P < 0.05$), and the highest proportion of follicles was found close to the small curvature of the ovary, near the ovulatory fossa, with 41.3% of the follicles (625/1514; SD=0.49); the intermediate region had 36.9% of the follicles (559/1514; SD=0.48) and the smallest follicular density was found in the external region, close to the ovarian greater curvature with 21.8% (330/1514; SD= 0.41). This study demonstrates that equine preantral follicles are concentrated in specific regions in the ovary, and a better understanding of this aspect can increase the efficiency of protocols for isolation and culture of preantral follicles in is species.

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A042 Folliculogenesis, Oogenesis and Ovulation

Effect of diet on *in vivo* embryo production of non-lactating and non-pregnant Holstein cows

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Keywords: embryo, energy source, superovulation.

The aim of the study was to evaluate the effect of two isocaloric and isonitrogenous diets, a corn-based and a citrus-pulp based diet (both mixed with sugarcane bagasse), on the quality and quantity of embryos produced by 25 non-lactating and non-pregnant Holstein cows. The hypothesis is that a diet generating higher proportion of propionate relative to acetate in the rumen, as citrus pulp, would have positive effects on embryo production. The study was performed in a crossover design with two 70-d long periods each. Two superovulatory (SOV) treatments were performed on each period with a 35-d interval between them, in order to evaluate the acute and chronic effects of energy sources. An adaptation diet was provided for 14 d before the beginning of the experiment and for more 14 d between the two periods. Diet was balanced for maintenance and was offered individually at 1% of body weight in dry matter. Cows were weighed every 14 d. The protocol used for SOVs included eight decreasing doses of FSH (Folltropin-V, Bioniche, Canada), 300 mg i.m in total. Frozen semen of three Holstein bulls, which were balanced between treatments, was used. Seven d after AI the embryos were collected according to the procedure described by Castro Neto et al. (Theriogenology, v.63, p.1249-1255, 2005) and classified according to the stage of development (Souza et al., Animal Reproduction, v.4, p.70-76, 2007) and quality: Grade I and II (freezable), Grade III, degenerate and unfertilized oocytes (UFO). Ultrasonography evaluations were performed at the end of the SOV protocol to count the number of follicles ≥8 mm. Data were evaluated by the ProcMixed of SAS, with a 0.05 significance level. There was no change on body weight throughout the experimental period. No difference was detected on the number of freezable embryos per cow between corn and citrus pulp treatments (2.5 ± 0.79 vs. 3.4 ± 0.77, respectively; $P = 0.24$), Grade III embryos (0.8 ± 0.22 vs. 0.5 ± 0.22; $P = 0.22$), viable embryos (Grades I-III; 3.6 ± 1.01 vs. 4.0 ± 0.99; $P = 0.96$), degenerate (2.3 ± 0.48 vs. 2.4 ± 0.47; $P = 0.85$) and UFO (5.0 ± 1.11 vs. 3.8 ± 1.09; $P = 0.21$). The recovery rate was 60.5 ± 5.86 vs. 56.4 ± 5.77% ($P = 0.53$) and the average embryo development stage was 4.3 ± 0.08 and 4.3 ± 0.08 ($P = 0.85$) for corn and citrus pulp, respectively. Regardless of treatment, the second SOV of each period (chronic effect) resulted in a lower proportion of freezable embryos compared to the first SOV (17.5 ± 5.39 vs. 33.1 ± 5.14%; $P = 0.003$), viable embryos (30.0 ± 6.83 vs. 39.3 ± 6.59%; $P = 0.05$) and UFO (37.4 ± 6.23 vs. 53.1 ± 5.88%; $P = 0.02$). The first period had a greater number of ovulated follicles per cow compared to the second (18.5 ± 1.96 vs. 15.2 ± 1.95; $P = 0.04$). It was concluded that there was no effect of diet on the production and quality of the embryos.

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A043 Folliculogenesis, Oogenesis and Ovulation

Effect of different concentrations of EGFon *in vitro* culture of equine ovarian follicles

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Keywords: EGF, equine, *in vitro* culture.

The aim of this study was to evaluate the effect of different concentrations of EGF (Epidermal Growth Factor) on in vitro culture of equine preantral follicles. Ovaries (n=5) obtained at a local slaughterhouse from mares at seasonal anestrus were washed in PBS and alcohol 70 and transported in PBS plus penicillin (200 IU/mL) and streptomycin (200 IU/mL). The inner portion of ovary was divided into 11 fragments of 3x3x1 mm. One fragment of each ovary was immediately fixed in Bouin (control group, D0). The remaining 10 fragments were individually cultured in 24-well culture plates containing 1 mL MEM (Gibco BRL, Rockville, MD, USA) (osmolarity 300 mOsm/L, pH 7.2) supplemented with penicillin (100 IU/mL), streptomycin (100 mg/mL), bovine serum albumin (1.25 mg/mL- Gibco BRL, Rockville, MD, USA), ITS (Insulin- 6.25 g/mL, transferrin - 6.25 g/mL, Selenium - 6.25 ng/mL), pyruvate (0.23 mM), glutamine (2 mM) and hypoxanthine (2mM). This medium was referred as MEM +. Culture was performed for 2 or 6 days with medium change every 2 days. Medium was supplemented with different concentrations of EGF (10, 50, 100 and 200 ng/mL). After culture, fragments were fixed in Bouin and processed for histology. Follicles were classified according to the stage of development (primary or developing) and morphology (normal or degenerated). A total of 825 slides containing 3,300 tissue sections were evaluated. The statistical model used was Proportion test ($P < 0.05$). After two days of culture there was a higher proportion of viable follicles at a concentration of 100 ng/mL EGF (87.5%), while MEM had 44.4%; 10 ng/mL had 22.2%; 50 ng/mL had 46,4% and 200 ng/mL had 64.9%. We observed follicular development in all tested concentrations of EGF after two days of culture. EGF at 100 ng/ml provided the best results, with all follicles in development, while MEM had 50%, 10 ng/mL had 75%, 50 ng/mL had 69.2% and 200 ng/mL had 91.7%. After six days of culture, EGF dose did not alter follicular viability. Regarding the proportion of developing follicles after six days, the best results were obtained with EGF at 10 ng/mL and 50 ng/mL with all follicles classified as developing, and 200 ng/mL with 85.7% of developing follicles. Therefore, EGF at 100 ng/mL promoted the best viability at two days of culture, while there was no difference between treatments for this endpoint after six days of culture. Considering follicular development, EGF at 100 ng/mL was the most effective for two days, whereas the doses of 10, 50 and 200 ng/mL were most effective after 6 days of culture to promote development. We conclude that there is a dynamic demand for EGF in *in vitro* culture of equine preantral follicles.

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A044 Folliculogenesis, Oogenesis and Ovulation

Effect of increasing circulating insulin on follicular development of Holstein cows

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Keywords: follicular growth, Holstein, Insulin.

The aim of this study was to test the hypothesis that increased circulating insulin during the pre and post deviation period would increase the initial and final follicular development. Sixteen non-lactating and non pregnant Holstein cows were used. The estrous cycles were synchronized using an intravaginal P4 device of 1.9 g (CIDR®; Zoetis, SP, Brazil) and intramuscular (i.m.) 100 µg of GnRH (Gonadorelin diacetate; Cystorelin®, Merial, Canada) on day -12 of the protocol (random day of the estrous cycle). On day -5, 25 mg were administered i.m. of $PFG_2\alpha$ (Dinoprost tromethamine; Lutalyse®, Zoetis, SP, Brazil). On day -3, the intravaginal P4 device was removed and cows received 25 mg of $PGF_2\alpha$ im. On days 10 and 11 ± 1 of the estrous cycle all follicles > 5 mm were aspirated to synchronize the emergence of a new follicular wave. The second day of aspiration was considered D1 of the experiment, when treatments were initiated. For this, cows were divided into two treatments: water (control; C) or propylene glycol (P) provided orally in four daily doses of 300 mL every 6 hours for 3 consecutive days (D1 to D3, pre follicular deviation period), and another 3 consecutive days (D5 to D7; after follicular deviation period). The experimental design was a Latin square in a 2x2 factorial arrangement Thus, four groups were formed: 1) Group CC = water pre and post follicle deviation (n = 16); 2) Group CP = water pre and propylene glycol post follicle deviation, respectively (n = 16); 3) Group PC = propylene glycol pre and water post follicle deviation, respectively (n = 16); and 4) Group PP = propylene pre and post follicle deviation (n = 16). Blood samples were taken 0 (immediately before), 15, 30, 60 and 120 minutes after propylene glycol for circulating insulin. Ovarian ultrasonography examinations were performed daily for evaluation of follicular dynamics. Statistical analysis was performed by the MIXED procedures of SAS. Plasma insulin concentrations were higher for groups receiving propylene glycol compared to controls (0, 15, 30, 60 and 120 min: 17.5 ± 1.4; 26.3 ± 1.4; 31.2 ± 1.4; 21.8 ± 1.4, 16.9 ± 1.5 vs.12.1 ± 1.5; 11.6 ± 1.5; 11.2 ± 1.5; 10.8 ± 1 5; 11.1 ± 1.5; $P \leq 0.05$). Despite this, there was no effect of circulating insulin increase ($P > 0.05$) on the rate of pre deviation (1.5 ± 0.14; 1.3 ± 0.15; 1.5 ± 0.14; 1.4 ± 0.15 mm/day) and post deviation (1.2 ± 0.13; 1.4 ± 0.14; 1.4 ± 0.13; 1.4 ± 0.13 mm/day) follicular growth and ovulatory follicle size (15.5 ± 0.56; 161 ± 0.55; 15.6 ± 0.52; 15.6 ± 0.54 mm) for CC, CP, PC and PP groups, respectively. We did not confirm the hypothesis that increased circulating insulin during pre and post deviation period interfere on follicle development.

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A045 Folliculogenesis, Oogenesis and Ovulation

Effect of insulin-like growth factor (IGF-1) on the morphology and development of ovine preantral follicles cultured *in situ*

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Keywords: growth, oocyte, sheep.

In vitro studies have demonstrated that insulin-like growth factor-1 (IGF-1) plays important roles in folliculogenesis, such as maintenance of survival after culture of caprine ovarian tissue (MARTINS et al., Animal Reproduction, v.7, n.4, p.349-361, 2010) and growth of isolated follicles in cows (GUTIERREZ et al., Biology of Reproduction, v. 62, p. 1322-1328, 2000). However, there is no report on the effect of different concentrations of IGF-1 on the *in vitro* development of ovine preantral follicles enclosed in ovarian tissue. Thus, the aim of this study was to evaluate the effect of IGF-1 on the morphology and activation *in vitro* of preantral follicles enclosed in ovine ovarian tissue. Ovaries (n=10) from adult mixed-breed sheep were collected at a local slaughterhouse and transported to the laboratory. The ovarian cortex was divided in fragments and one of them was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured *in vitro* for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with insulin, transferrin, selenium, hypoxanthine, glutamine, ascorbic acid and BSA (α-MEM+ - control medium) or in α-MEM$^+$ added by different concentrations of IGF-1 (1, 10, 50,100 or 200 ng/mL). At the end of the culture, morphological analysis of preantral follicles was performed through histology, and they were classified as normal or atretic according to the absence of presence of cytoplasmic shrinkage, nuclear pycnosis and/or disorganization of granulosa cells, as well as classified as primordial or growing follicles (intermediate, primary and secondary follicles). The percentage of normal, primordial and growing follicles were compared by ANOVA and Tukey's test ($P < 0.05$). The results showed that after 7 days of culture, there was a significant reduction in the percentage of morphologically normal follicles in all treatments compared to the fresh control. However, concerning follicle survival, no significant differences ($P > 0.05$) were observed between α-MEM$^+$ and IGF-1 concentrations. In comparison with fresh control, it was observed a significant reduction in primordial follicles and an increase in developing follicles in all treatments. Moreover, treatment with 100 ng/mL IGF-1 promoted higher ($P < 0.05$) follicular activation, compared to α-MEM$^+$. In conclusion, the concentration of 100 ng/mL IGF-1 maintained survival and promoted activation of ovine preantral follicles cultured *in situ*.

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A046 Folliculogenesis, Oogenesis and Ovulation

Effect of IGF-1 SnaBI polymorphism on reproductive parameters and metabolic parameters in dairy cows

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Keywords: fertility, IGF, Snp.

Insulin-like growth factor 1 (IGF-1) is associated with increased follicular estradiol production, stimulating the return of postpartum ciclicity (Butler et al., 2004). Therefore, it is possible that genetic variants of the IGF-1 gene can improve reproductive efficiency of postpartum cows. The aim of this study was to evaluate the effect of polymorphisms in the IGF-1 gene on reproductive parameters, and milk production of Holstein dairy cows. Genotyping was performed by electrophoresis of the PCR product after digestion with the enzyme SnaBI. Holstein cows (n=75) from 21 days prepartum up to 210 days in milk (DIM) were used in the study. These cows were submitted to an OvSynch-TAI protocol at 55 DIM, and the protocol was repeated in cows diagnosed as non-pregnant at 30 and 60 days after AI. Milk samples were collected twice per week for determining ovulation. Progesterone levels above 1 ng/mL in two consecutive samples indicated ovulation. Days from calving to first ovulation (CFO) and the calving to conception interval (CCI) were evaluated. Serum concentrations of IGF-1 and β-hydroxybutyrate (BHBA) were measured in samples collected at -21, 0, 7, 21 and 60 DIM. Data were analyzed using the GLM procedure of SAS. Genotype distribution was 14.7% for the TT genotype, 48% for CT and 37.3% for CC. Circulating IGF-1 levels were 79.2 ± 9.9, 66.5 ± 5.2 and 56.6 ± 5.9 ng/ml for TT, TC and CC genotypes, respectively (P=0.05). The CFO interval for TT, TC and CC cows was 19.9 ± 4.2, 30.6 ± 2.3 and 30.4 ± 2.5 days, respectively, indicating a shorter interval (P<0.05) for TT cows, which had the highest levels of IGF-I. A linear effect (P<0.05) was observed among genotypes for the CCI, which was 76.9 ± 12.6, 96.9 ± 6.8 and 111.7 ± 7.8 for TT, CT and CC, respectively. Cows from the TT genotype had a shorter CCI that may be associated with earlier return to postpartum cyclicity and higher serum IGF-I levels. The TT cows had lower serum BHBA values than cows with TC and CC genotypes, 5.0 ± 1.4, 8.2 ± 0.7 and 8.1 ± 0.8 mg/dL (P<0.05), respectively. Milk production was not different between groups (P>0.05). In conclusion, the IGF-1 SnaBI polymorphism (TT) was associated with reduced CFO and CCI in dairy cows.

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A047 Folliculogenesis, Oogenesis and Ovulation

Effect of the type of fixative and time of fixation on the morphology of equine preantral follicles

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Universidade Estadual de Londrina.

Keywords: equine, fixatives, ovarian tissue.

The objective of this study was to investigate the efficacy of tissue fixatives Bouin, Carnoy and formaldehyde in equine ovarian fragments. Ovaries (n=4) from mixed breeds mares were obtained at a local slaughterhouse and transported to the laboratory in a thermo-recipient at 20ºC. Immediately after collection ovaries were washed with modified PBS solution (Cultilab®, Campinas-SP, Brazil) and divided into nine fragments with approximately 5x5x1 mm. Ovarian fragments were immersed in Bouin (B), Carnoy (C) or Formalin 10% (F) for 6, 12 or 24 hours. Each fragment was individually immersed 20 mL Falcon tubes containing 20 times the volume of fixative solution. After this period, they were kept in 70% ethanol for 24 hours. Each procedure was performed in four replicates. For histological analysis, samples were dehydrated in increasing concentrations of alcohol, diaphanized in xylene and embedded in paraffin. Five µm thick serial sections were obtaneid with a rotating microtome (Leica® type, Wetzlar, Germany) and stained with periodic acid-Schiff (PAS) and hematoxylin. A total of 540 slides with 1,620 sections were evaluated and 465 preantral follicles were counted and classified as normal or degenerated. Degeneration was determined by the presence of at least one of the following aspects: retraction of the cytoplasm, pyknotic nucleus, cytoplasmic vacuoles, displacement of granulosa cells and disruption of the basal membrane. The statistical model was logistic regression ($p < 0.05$). The Carnoy fixative for 24 hours provided best integrity of ovarian follicles (53.3%; 32/60) compared to the others. Boiun for 24 hours was the worst treatment (19.1%; 9/47). The other treatments showed the following results; C12h 50% (30/60), C6 H 40% (24/60), F24h 37.8% (17/45), F12h 35.1% (13/37), F6h 32% (16/50), B12h 30.5% (18/59) and B6h 24.4% (11/45). Fixation with Carnoy for 24 hours provided best integrity of equine preantral follicles.

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A048 Folliculogenesis, Oogenesis and Ovulation

Effects of a high-fat and energy diet on ovarian gene expression in young and aged female mice

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Keywords: aging, AMH, IGF.

Studies indicate that high-fat diets can have adverse effects on ovarian activity and that aging can reduce female fertility due to decreased quality and size of the oocyte ovarian reserve. The PI3K/AKT1 signaling pathway and the FOXO3a transcription factor are important regulators of cell proliferation and survival and are involved in the activation of primordial follicles. The aim of the current study was to determine the effects of a high-fat and energy diet on ovarian gene expression (AMH, IGF-1, AKT1, PI3K, MTOR, BMP15, CAT, SIRT1 e FOXO3) in young and aged female mice. Twenty female mice (C57BL/6) at four and thirteen months of age were used (young: n = 10 and old: n = 10). Animals were divided into four groups: young/control diet (YC); young/high fat diet (YH); old/control diet (OC) and old/high-fat diet (OH). Both diets contained 14.1% crude protein, but the control diet had 75.9% carbohydrate, 10.0% fat and 3,061 kcal/100g of energy, while the high-fat diet contained 54.6%; 32.7% and 4,402/100g kcal, respectively. Females were fed the diets during 55 days, intake was measured every two days and body weight measured weekly. After euthanasia, ovaries were collected, RNA was extracted using the Trizol method (Trizol, Invitrogen, USA), RNA was converted to cDNA (Biorad, , Hercules, CA, EUA) and the expression of target genes measured by qRT-PCR (Applied Biosystems, Foster City, CA, USA) using the β2 - microgobulina as the endogenous control. For statistical analysis data were compared by two-way ANOVA (GraphPad Software Inc., La Jolla, CA, USA) for testing the effect of age, diet and its interaction. AMH was 67% less expressed in old than in young females ($P<0.05$). Furthermore, there was a 46% reduction in the expression of IGF-1 in females fed the high-fat diet ($P<0.05$), as well as lower food intake when compared to mice fed the control diet ($P<0.05$; 2.9 ± 0.1 and 3.9 ± 0.1). Previous studies suggest that IGF-1 may be involved in the premature depletion of the ovarian reserve (Schneider et al., 2015; J Ovarian Res. 7: 120). Females fed the high-fat diet had increased body weight gain compared to controls ($P<0.05$; 28% and 9% gain, respectively). In conclusion, there was a reduction in IGF-1 expression in the ovary of females fed the high fat diet, which may be a factor modulating ovarian aging.

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A049 Folliculogenesis, Oogenesis and Ovulation

Effects of fibroblast growth factor 8 on meiosis progression and cumulus expansion of bovine cumulus-oocyte complexes submitted to *in vitro* maturation with amphyregulin

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Keywords: AREG, FGF8, oocyte maturation.

Fibroblast growth factor 8 (FGF8) is expressed by the bovine oocyte and appears to be involved in the mechanisms underlying meiotic arrest in mice via regulation of NPR2 expression in cumulus cells and, consequently, levels of cGMP. Recent studies suggest that replacement of FSH by amphyregulin (AREG) during in vitro maturation (IVM) may improve embryo development. The objective of this study was to assess the effects of FGF8 on cumulus expansion and meiosis progression in bovine cumulus-oocyte complexes (COCs) undergoing IVM with AREG. Bovine COCs were aspirated from slaughterhouse ovaries and cultured (grade 1 and 2) in groups of 20 in 400 µL TCM 199 supplemented with BSA (0.4%), sodium pyruvate (22 µg/mL), amikacin (75 µg/mL), AREG (100 ng/mL) and FGF8 at 0, 10 and 100 ng/mL, at 38.5 °C with 5% CO2 and maximal humidity. The degree of expansion was visually assessed after 22h of IVM (n=5 replicates) using a subjective method (degrees 1: poor expansion, 2: partial expansion and 3: full expansion), while meiosis progression was assessed at 6 (n=4 replicates) and 22h (n=5 replicates) of IVM. In order to assess nuclear maturation, oocytes were mechanically denuded, fixed, stained with Hoechst 33342 and examined under a fluorescence microscope to be classified as at germinal vesicle (GV) or germinal vesicle breakdown (GVBD) at 6h, or at MI (metaphase I) or MII (metaphase II) at 22h of IVM. Data were expressed as percentages and transformed to arcsine before testing effects of treatments by ANOVA followed by the Fisher Protected test. Differences were considered significant when $P < 0.05$. FGF8 at 100 ng/mL increased the percentage of GV oocytes in relation to the control at 6h (37.4 ± 7.9%, 49.8 ± 5.6% and 59.3 ± 4,8% of GV oocytes for 0, 10 and 100 ng/mL FGF8, respectively), but did not change MI (22,7 ± 2,4%; 16±5% and 25,3±8,9%) and MII rates (77.2 ± 2.3%; 83.8 ± 5.03% and 74.5 ± 8.9% for 0, 10 and 100ng/ml FGF8, respectively), nor expansion (63.3 ± 15.8%; 56.6 ± 22.1% and 56.6 ± 27.4% of grade 3 COCs for 0, 10 and 100 ng/mL FGF8, respectively) at 22h. We conclude that addition of FGF8 to the culture medium is capable of retarding germinal vesicle breakdown in oocytes submitted to IVM with AREG without compromising meiosis completion at the end of culture.

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A050 Folliculogenesis, Oogenesis and Ovulation

Effects of follicle stimulating hormone (FSH) and amphiregulin (AREG) on meiosis dynamics of oocytes undergoing *in vitro* maturation

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Keywords: AREG, FSH, meiosis.

Oocyte meiosis is arrested at prophase I during early folliculogenesis and is resumed after the ovulatory LH surge, which induces germinal vesicle breakdown (GVBD) and progression of nuclear maturation to metaphase II (MII). Resumption of nuclear maturation is associated with decreases in oocyte transcription/translation and intracellular communication within the cumulus-oocyte complex. Therefore, precocious meiosis resumption may compromise oocyte fertility. The influences of LH in ovulatory follicles and of FSH during in vitro maturation (IVM) are mediated by EGF-like growth factors, including amphiregulin (AREG), which are produced by mural granulosa and cumulus cells. Recent studies suggest that replacement of FSH by AREG in IVM may improve oocyte developmental competence. This study aimed to assess meiosis dynamics in oocytes cultured with FSH or AREG. We tested the hypothesis that the direct stimulation with AREG speeds up nuclear maturation. Cumulus-oocyte complexes (COCs) were aspirated from bovine ovaries obtained at a slaughterhouse, and submitted to IVM for 6 hours in TCM 199 supplemented with BSA (0.4%), amikacin (75µg/mL), pyruvate (22µg/mL) and FSH (1µL/mL; n=81) or AREG (100ηg/mL; n=59). Cultures were performed in 7 replicates. After culture, oocytes were mechanically separated from cumulus cells, fixed with paraformaldehyde 4%, stained with Hoescht 33342 and examined under a fluorescence microscope to assess meiosis. Data were transformed to arcsine and groups compared by the Student t test, considering values of $P<0.05$ as significant. Supplementation with AREG resulted in a lower percentage of germinal vesicle oocytes (39.12% ± 6.6% vs. 61.06 ± 5.12; P=0.04) and a higher percentage of oocytes in MI (23.59% ± 5.5 vs. 9.93 ± 1.04; P = 0.04) in comparison with FSH. The percentage of oocytes at GVBD did not differ between treatments (P = 0.1). The present data indicate that AREG induces nuclear maturation more rapidly than FSH.

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A051 Folliculogenesis, Oogenesis and Ovulation

Expression of LH receptor in bovine *Cumulus oophorus* cells is modulated by follicular diameter and the gonadotropins stimulus

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Keywords: cumulus cells, LH receptor, oocyte.

Reproduction in mammals depends on the pulsatile release of the gonadotropins LH and FSH, which act together to regulate the function of the gonads. In the ovaries, LH influences follicular development until ovulation and this depends on the interaction with its receptor (LHR). However, there is no consensus regarding the use of LH during the in vitro maturation (IVM) of cumulus-oocyte complexes (COC) in cattle. Reports in the literature differ on mRNA expression of LHR in oocytes and cumulus oophorus cells (CC). Thus, the aim of this study was to characterize the expression of the LHR gene in CC and oocytes from bovine follicles at different diameters and after IVM. Ovaries were collected at a commercial abattoir and follicles were dissected and separated into two groups for experiment 1, G1: 3 to 6mm and G2: >6 to 10mm. On a Petri dish, follicles were individually ruptured with 18G needles in order to obtain their respective COC. Pools of 25 COC were used per experimental group from which CC were separated mechanically from the oocytes and stored. In experiment 2, follicles ranging 3-8mm were aspirated and the obtained COC were matured in vitro (groups of 25 COC) in droplets (100 μL in mineral oil) of IVM medium (TCM199, sodium pyruvate, 0.4% BSA and amikacin) at 38.5°C and 5% CO2 in air for 24h. The experimental groups were divided into control (IVM medium); rhLH (IVM+0.01 rhLH); and rhFSH (IVM+0.01 rhFSH) from which CC were separated from oocytes and stored. All samples were subjected to total RNA extraction using Trizol and reversely transcribed with High-capacity kit. For real-time PCR, we used primers targeting a fragment of the LHR gene not subjected to alternative splicing, and PPIA was used as the reference gene. All experiments were performed in triplicate. Data were analyzed by ANOVA followed by Tukey test when necessary (5% significance level). LHR mRNA was not detected in oocyte samples (experiments 1 and 2). Experiment 1 - LHR mRNA was detected in both groups of CCs from follicles at different sizes, and mRNA levels did not differ between these groups ($P > 0.05$). Experiment 2 – After IVM, LHR mRNA was detected in rhLH and rhFSH groups ($P > 0.05$) and was absent in the control group. The data from experiment 1 suggest that follicle growth does not appear to affect abundance of LHR mRNA in CC. In experiment 2, the abundance of LHR mRNA in CC was only observed in groups treated with gonadotropins (rhFSH and rhLH) suggesting that FSH and LH stimulate LH receptor expression in cumulus cells.

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A052 Folliculogenesis, Oogenesis and Ovulation

Expression of activin receptors during follicular deviation and luteolysis in cattle

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Keywords: corpora lutea, follicular deviation, TGFbeta.

Several signaling factors act on theca and granulosa cells and oocytes to promote follicular proliferation and differentiation in the ovary. In this context, the transforming growth factor β (TGFβ) superfamily has been studied. Activin receptors type II were shown to modulate signaling of TGFβ members and betaglycan is a co-receptor that enhances the effect of ligands of type II receptors. The objective of this study was to quantify mRNA levels of activin receptors type I (ACVR1A, ACVR1B), type II (ACVR2A and ACVR2B) and betaglycan during follicular deviation and luteolysis in cattle. Ovaries were collected by colpotomy and granulosa cells from the two largest follicles were isolated before (day 2; F1 and F2; n=4), during (day 3; dominant follicle (DF) and subordinate (SF); n=4) or after (day 4; DF and SF; n=6) follicular deviation. For luteal cells analysis during luteolysis, corpora lutea were dissected from the ovary at 0 (control), 2, 12, 24 and 48 h after treatment with PGF2α, which was injected 10 days after estrus detection (n=4-5 per group). The deviation model was validated by assessing mRNA levels of CYP19A1, which were higher in DFs ($P<0.05$) during and after follicular deviation. To validate the luteolysis model, serum progesterone levels were shown to decrease at 2 h, and reached basal levels at 24 h post-PGF treatment, which confirmed functional luteolysis. Data were tested for normal distribution using the Shapiro-Wilk test, normalized when necessary and submitted to ANOVA. ACVR2A mRNA levels were higher ($P<0.05$) in F1 follicles before deviation (day 2) compared to DFs after deviation (day 4). ACVR1B mRNA abundance decreased in both healthy (F1 and DFs) and atretic (F2 and SFs) follicles from day 2 to day 4. However, ACVR1A and ACVR2B mRNA levels did not change during follicular deviation. Betaglycan mRNA abundance was higher ($P<0.05$) in SFs than DFs at the expected time of deviation (day 3). During luteolysis, ACVR2A mRNA levels increased at 2 h ($P<0.05$) but decreased at 48 h post-PGF treatment. These results indicate that type II activin receptors are regulated during follicular deviation and luteolysis, and betaglycan at the expected time of follicular deviation, which suggests a potential role in the regulation of these ovarian processes.

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A053 Folliculogenesis, Oogenesis and Ovulation

Epidermal growth factor (EGF) induces cell proliferation during *in vitro* culture of ovine preantral follicles

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Keywords: activation, mitosis, oocyte.

Efficient *in vitro* culture systems of ovarian preantral follicles can promote oocyte growth and proliferation of somatic cells. After *in vitro* culture of ovine ovarian tissue, it was observed that control medium (α-MEM$^+$) alone or added by 1 ng/mL Epidermal Growth Factor (EGF) can promote the initiation of primordial follicle development, process also called follicular activation (SANTOS et al., 2013, Anais da XXVII Reunião Anual da SBTE). However, there is not information about oocyte growth and proliferation of granulosa cells. Thus, the aim of the present study was to determine the effect of EGF (1 ng/mL) on oocyte diameter and granulosa cell proliferation after *in vitro* culture of ovine ovarian tissue. After collection of ovine ovaries (n=10) in the slaughterhouse, ovarian cortex was fragmented and one fragment was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured *in vitro* for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with ITS (10 µg/mL insulin, 5.5 µg/mL transferrin, 5,0 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL bovine serum albumine (BSA) and 50 µg/mL ascorbic acid (α-MEM$^+$ - control medium) or in α-MEM$^+$ added by 1 ng/mL EGF. After culture, the fragments were destined to histology, the oocyte diameter was analyzed and immunohistochemistry was performed to evaluate Proliferating Cell Nuclear Antigen (PCNA; 1:500; Santa Cruz Biotechnology; Santa Cruz, CA, EUA). Proliferating or PCNA-positive cells (brown) were counted in the sections and expressed as percentages. The diameter was analysed by ANOVA and the percentages of PCNA-positive cells were compared by Qui-square test ($P<0.05$). There was no influence of α-MEM$^+$ or EGF on oocyte diameter after *in vitro* culture. Concerning cell proliferation, medium supplementation with EGF significantly increased PCNA-positive cells (55.0%) in relation to the fresh control (13.0%) and control medium (5.0%). In conclusion, 1 ng/mL EGF promotes proliferation of granulosa cells in preantral follicles during *in vitro* culture of ovine ovarian tissue.

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A054 Folliculogenesis, Oogenesis and Ovulation

Improvement on the viability of preantral *Bos indicus* follicles cultured *in vitro* by addition of follicle stimulating hormone (FSH)

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Keywords: *Bos indicus*, FSH, *In vitro* culture.

The aim of this study was to evaluate the effect of FSH addition in the medium for *in vitro* culture of preantral follicles of *Bos indicus* females. Ovaries (n=10) were collected at a local slaughterhouse from five adult cycling *Bos indicus* cows (Nelore) with body condition score from 2.5 to 3.5 (range 0-5). After collection, ovaries were washed in 70% ethanol and PBS (Embriolife®, Vitrocell, Brazil). The surrounding tissue was removed and ovaries were sectioned longitudinally. The ovarian cortex was divided in 3x3x1 mm fragments. One fragment per animal was immediately fixed in Bouin (non-cultured control, D0). The other fragments (n=8) were individually cultured in 24-well culture plates containing 1 ml of minimum essential medium (MEM, Gibco BRL, Rockville, MD, USA; osmolarity 300 mOsm/l, pH 7.2) supplemented (MEM+) with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin, and 6.25 ng/ml selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxantina, 1.25 mg/ml bovine serum albumin (BSA Gibco BRL, Rockville, MD, USA), 20 IU/ml penicillin and 200 mg/ml streptomycin. Unspecified reagents were obtained from Sigma (St. Louis, MO, USA). MEM + medium was added to different concentrations (50, 100 and 200 ng/ml) FSH (Folltropin®, Bioniche Canada Inc, Ontario, Canada). The *in vitro* culture medium was tested during two (D2) or six (D6) days. Every two days, the culture media was replaced by fresh aliquots. For the analysis of integrity and degree of follicular development we used histology with periodic acid-Schiff (PAS) and hematoxylin staining. The classification was based on the evaluation of follicular development stage (primordial, primary and secondary) and morphological integrity as normal or degenerated. Data were submitted to ANOVA ($p \leq 0.05$). We evaluated 2,250 preantral follicles (normal or degenerated), being 772 primordial and 1,478 in development. After two days of culture, FSH at 100 ng/ml provided a higher proportion (51.2%; 128/250) of follicles morphologically intact when compared to the other groups: 27.2% (68/250) to MEM; 30.4% (76/250) to 50 ng/ml; 45.2% (113/250) to 200 ng/ml ($p<0.05$). Higher percentage of developing follicles was also obtained with FSH at 100 ng / ml (91.8%, 112/122). After six days of culture, 100 and 200 ng/ml of FSH provided a higher percentage of morphologically intact follicles (40.4%, 101/250 and 36.8%, 92/250, respectively) compared to 50 ng/ml. There was no difference between groups for the rate of development at D6 ($p>0.05$). We conclude that MEM+ supplemented with 100 ng/ml FSH for two or six days of *in vitro* culture was the most effective treatment to provide development and morphological integrity of preantral follicles from *Bos indicus* cows.

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A055 Folliculogenesis, Oogenesis and Ovulation

Intrafollicular injection with PPARγ agonist on follicle deviation in cows

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Keywords: cows, follicle deviation, PPAR.

Gamma receptor peroxisome proliferator – activated (PPARγ) is a member of the PPAR nuclear receptors family, and these receptors are expressed in reproductive tissues from different species. PPARs have been shown to control apoptosis and cell cycle and to affect estradiol synthesis and metabolism. In rats, the expression of this receptor increased with follicular growth and decreased after the LH surge. In sheep and buffalos, PPARγ expression is primarily restricted to granulosa cells of developing follicles, and its expression is higher in small follicles. This receptor can also be activated by synthetic ligands such as Thiazolidinediones (TZD), a family of drugs that are insulin-sensitizers and PPARγ agonists. Our hypothesis is that PPARγ signaling is related with follicular atresia and may be involved in follicle deviation in monovulatory species. To test this hypothesis, the aim of this study was to evaluate if intrafollicular injection of a PPARγ agonist (TZD) inhibits dominant follicle growth. *Bos taurus* cows had the emergence of a new follicular wave induced by treatment with a progesterone releasing intravaginal device (1 g of the progesterone, DIB-Intervet Schering Plough, Brazil) and injection of 2 mg estradiol benzoate (i.m.; Genix, Anápolis, Brazil). Four days later, the progesterone device was removed, cows received intramuscularly (i.m.) injections of $PGF_2\alpha$ analogue (cloprostenol, 250 µg; Schering-Plough Animal Health, Brazil) and ovaries were monitored once a day by transrectal ultrasonography using an 8 MHz linear probe (Aquila Vet scanner, Pie Medical, Netherlands). When the largest follicle reached between 7 and 8 mm in diameter (ten cows), tcows were randomly assigned to receive an intrafollicular injection of 50 µM TZD (n=5) or PBS (n=5). The intrafollicular injection volume was adjusted according with follicular size in order to obtain a final concentration of 50 µM TZD in all follicles. The injected follicle was monitored daily by ultrasonography for three days after the injection as described by Ferreira et al. (Reproduction, 134, 713-9, 2007). The average sizes of the PBS injected follicles were 7.5±0.1 mm, 8.3±0.5 mm, 9.9±0.5 mm and 10.9±0.3 mm at 0, 24, 48 and 72h after treatment, respectively. TZD injected follicles stopped growing after injection and the follicular size curve was statistically different in relation to the control group (7.6±0.1 mm, 6.4±0.3 mm, 5.2±0.4 mm and 5.0±0.3 mm at 0, 24, 48 and 72h after treatment, respectively). Treatment with TZD inhibited follicular growth in all cows (5 out 5) and follicles injected with PBS continued growing (5 out 5). In conclusion, the increase in PPARγ signaling inhibited follicular growth and may be involved in the selection of the dominant follicle in cattle.

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A056 Folliculogenesis, Oogenesis and Ovulation

Melatonin in *in vitro* maturation and their effect on the expression of antioxidant and apoptosis related genes of murine cumulus-oocyte complexes

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Keywords: IVM, melatonin, mice.

Melatonin (MLT) is a hormone synthesized primarily in the pineal gland and participates in the control of the circadian cycle and reproductive seasonality in some animal species. In addition to these functions, MLT also acts as a powerful antioxidant and antiapoptotic factor. The aim of this study was to assess the effect of the addition of MLT during *in vitro* maturation (IVM) on nuclear maturation and expression of genes related to antioxidant and apoptotic activity in murine *cumulus* cells (CC) and oocytes (OO). Female F1 hybrids (n=20; C57BL/6 x CBA) were subject to intraperitoneal injection of eCG (5IU/0.1 mL/animal) and after 48h, the *cumulus*-oocyte complexes (COCs) were collected. COCs (n=25 per group/treatment) were randomly selected and *in vitro* matured with MLT (10^{-9}, 10^{-6} and 10^{-3} M) or 0.5 µg/mL FSH (control) for 17h in an incubator at 37ºC and 5% CO_2 in air. Only MLT and not its association with FSH was used in order to evaluate their individual action on IVM and gene expression. Maturation rate was assessed according to the presence of the first polar body under an inverted microscope. Gene expression was evaluated by real-time quantitative PCR (4 replicates) for BAX and BCL2L1 (apoptosis) and GPX1, SOD1 and SOD2 (antioxidant) in CC and OO. As endogenous control, the geometric mean of H2AFZ and HPRT1 genes was used. Statistical analyses were performed by ANOVA followed by Tukey's test (4 replicates) with 5% significance level. No differences were detected ($P>0.05$) between groups matured with MLT at different concentrations (56.0%, 56/100; 53.7%, 51/95; and 48.9%, 43/88; for 10^{-9}, 10^{-6} and 10^{-3} M MLT, respectively) compared with the control group (57.3%, 55/96; FSH). The expression of GPX1 and SOD1 in CC was increased by MLT at 10^{-9} and 10^{-6} M ($P=0.0006$ e 0.0045, respectively). For OO, the group treated with 10^{-6} M MLT showed increased expression ($P=0.0208$) of the BAX pro-apoptotic gene. For the other genes, there was no difference between treatments ($P>0.05$). In conclusion, under the conditions studied, MLT was unable to improve the IVM rate, but alone was as efficient as FSH in promoting maturation of murine oocytes, indicating its potential effect on stimulating meiosis. In the real-time quantitative PCR analysis, the group treated with melatonin at 10^{-9} M presented increased expression of an antioxidant enzyme, suggesting the ability to enhance antioxidant action in CC.

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A057 Folliculogenesis, Oogenesis and Ovulation

Fibroblast growth factor 10 (FGF10) and bone morphogenetic protein 15 (BMP15) inhibit apoptosis in cumulus cells from bovine cumulus-oocyte complexes undergoing *in vitro* maturation

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Keywords: apoptosis, cumulus cells, FGF10.

Oocyte secreted factors (OSFs) regulate apoptosis in cumulus cells (CC). FGF10 and BMP15 are OSFs that enhance cumulus expansion and embryo development when added to the IVM medium. Progesterone signaling is anti-apoptotic in CC and essential for oocyte developmental competence. The aim of this study was to assess the influence of BMP15 and FGF10 alone or combined on CC apoptosis, as well as on expression of apoptosis related genes (FAS, PUMA, BAX, BCL2, BIRC4, MDM2 and nPR) and progesterone production in bovine COCs undergoing IVM. Groups of 20 COCs (grades 1 and 2) from 3-8mm follicles of abattoir ovaries were cultured for 22 hours in 450µL of maturation medium (TCM 199 containing Earle's salts supplemented with 0.4% BSA; 1µl/mL FSH; 22µg/ml sodium pyruvate, 75µg/ml amicacin) without growth factors (control group) or supplemented with FGF10 (R&D Systems, 10ng/mL), BMP15 (R&D Systems, 100ng/mL) or FGF10 and BMP15 combined (5 replicates for gene expression and progesterone; 8 replicates for apoptosis). After IVM, CC and oocytes were mechanically separated and CC were stained with propidium iodide (PI) and Annexin V- APC (BD Biosciences Pharmingen), and counted in a flow cytometer to quantify viable (PI-/A-), early apoptotic (PI-/A+), apoptotic (PI+/A+) and necrotic (PI+/A-) cells. Progesterone concentrations were measured in the culture medium by radioimmunoassay (Kodalmedical, IgAc). Abundance of mRNA encoding pro and anti-apoptotic factors was assessed by real time qPCR normalized to CYC-A. Effects of treatments were tested by ANOVA and means were compared by the Fisher protected test. FGF10 increased the percentage of viable cells (66±2.4, 81.9±3.2, 72±3.2 and 71.9±3.5 for control, FGF10, BMP15 and FGF10+BMP15, respectively), decreased the percentage of apoptotic cells (22.7±2.1, 7.2±1.1, 19.7±2.9 and 21.9±2.7) and increased the BCL2/BAX ratio (0.7±0.1, 1.2±0.1, 0.5±0.1 and 0.7±0.1). The combination FGF10+BMP15 decreased the percentage of early apoptotic cells (6.9±1.3, 7.7±2.3, 4.4±0.8 and 2.7±0.4 for control, FGF10, BMP15 and FGF10+BMP15, respectively), decreased FAS (1.3±0.21, 1.3±0.1, 0.98±0.22 and 0.81±0.17) and increased nPR (0.41±0.05, 0.64±0.15, 0.8±0.27 and 0.99±0.16) mRNA abundance. Progesterone production and expression of PUMA, BIRC4 and MDM2 were not affected by growth factors. The present data suggest an antiapototic role for FGF10 in the bovine COC by mechanisms involving regulation of BLC2/BAX ratio. Moreover FGF10 appears to interact with BMP15 to prevent early apoptosis during IVM by mechanisms involving suppression of FAS and increased nPR expression. The anti-apoptotic action of these OSFs on CC may account for their positive impact on oocyte developmental competence.

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A058 Folliculogenesis, Oogenesis and Ovulation

The chemokine receptor-2 (CCR2) plays a critical role on the follicular activation and preantral folliculogenesis and CCR2 deficiency leads in reduced fecundity

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Keywords: CCL2, CCR2, folliculogenesis.

Chemokines are cytokines with low molecular weight classically characterized by controlling the recruitment and activation of leukocytes during inflammation. The PI3K (Phosphoinositide-3-kinase) pathway is important for follicular activation and CCL2 chemokine (MCP-1) and its main receptor (CCR2), both highly expressed in mice ovaries, are modulated by PI3K levels. The study of the role of CCR2 in the reproductive system has clinical significance because CCR2 antagonists are under clinical trials for the treatment of autoimmune diseases. In recent studies performed by our group, we detected expression of CCR2 in mouse and human testicles, and observed that CCR2 deficiency caused a drastic reduction in daily sperm production. However, the role of this chemokine-receptor interaction in ovarian homeostasis is not known. The aim of the present study was to investigate CCR2 expression in wild-type mice ovaries (WT), and to quantify and compare the ovarian follicular population, recruitment rates, and follicular atresia in WT and CCR2-/ mice. Ovaries were collected from WT (n=10) and CCR2-/- (n=10) female (60 days-old) mice and processed for morphometric analysis, follicular quantification, immunohistochemistry, and western blotting. The mean number of litters per bred female and the number of pups per litter (during 1 year) were also recorded. All data were compared using t tests. The CCR2 protein was observed in the WT ovarian lysate and was not observed in the CCR2-/-, while β-actin expression (control) was observed in both groups. Immunohistochemical analysis revealed that CCR2 was not present in primordial follicles. However, this receptor was immunolocalized to oocytes included in activated follicles (primary, secondary, antral, and atretic). Although body and ovarian weight and gonadal-somatic index were not different between groups ($P>0.05$), CCR2 deficiency affected ovarian follicular population. CCR2-/- mice had an ovarian follicular reserve ~40% larger than WT ($P<0.01$). However, there was a reduction (~50%) in the number of preantral follicles ($P<0.01$), while the number of antral follicles was not different ($P>0.05$). Corroborating these findings, lower activation and follicular atresia index and fewer litters/bred female were observed in CCR2-/- ($P<0.01$). Overall, this study demonstrates for the first time that (i) CCR2 is a phenotypic marker and a potential regulator of follicular activation, (ii) CCR2 regulates mainly early stages of folliculogenesis, and (iii) CCR2 deficiency can lead to a significant reduction in fertility. Finally, this study indicates that the effects of CCR2 antagonists on folliculogenesis should be carefully studied before commercialization. CEUA 27/2014.

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A059 Folliculogenesis, Oogenesis and Ovulation

Is the NPPC/NPR2 system, described for murine model, applied to bovine antral follicles?

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Keywords: *In vitro* maturation, meiotic arrest, NPPC.

Recent studies in mice have demonstrated that the precursor of C-type natriuretic peptide (NPPC) and its receptor type 2 (NPR2) are essential for maintaining meiotic arrest in the oocyte and that estradiol, present in follicular fluid (FF), promotes and maintain expression of NPR2 in cumulus cells (CC), acting on the system NPPC/NPR2. The bovine FF influences nuclear maturation when added to the maturation media. In addition, extracellular vesicles (ECV), present in the FF, might contribute in the communication between ovarian somatic cells and oocyte. However, the presence of NPPC-NPR2 has not yet been identified in bovine ovary. Therefore, the objectives of this study are to elucidate the NPPC-NPR2 system in the bovine ovary and assess the influence of exogenous NPPC, estradiol and ECV on mRNA expression of PDE3 and NPR2, levels of cAMP in the oocyte and cGMP in CC and progression of meiosis I in bovine cumulus-oocyte complexes (COC). Five experiments were performed: 1) Immunoassay (ELISA) was used to detect and measure the concentration of NPPC in FF, lysate granulosa cells and ECV from groups with different follicular diameters (3-6mm;.>6-8mm and >8mm); 2) Immunolocalization of NPR2 receptor in bovine antral follicles; 3) COCs were cultured with exogenous NPPC, estradiol or ECV a) for 6 hours to assess mRNA expression of PDE3 in the oocyte and NPR2 in CC by RT-PCR and b) for 9 hours to evaluate meiosis progression by orcein staining and 4) Levels of cAMP and cGMP in COCs matured for 6 hours with exogenous NPPC or ECV were measured using commercial EIA kits (cAMP and cGMP, Format A PLUS, Biomol). Data were tested by ANOVA and means compared by Dunnett test at JMP 7.0 software (SAS Institute). Differences were considered significant when $P < 0.05$ and trend when $0.05 < P < 0.1$. NPPC was detected in higher concentrations in ECV, regardless follicular diameter. NPR2 was preferably located in mural granulosa cells. Estradiol decreased mRNA abundance of PDE3 in the oocyte and treatments did not influence the expression of NPR2 mRNA in CC. ECV and NPPC decreased degradation of cAMP (in the oocyte) and cGMP (in CC), respectively. All three treatments increased the percentage of COCs in GV stage. We conclude that NPPC, exogenously or contained in the ECV of bovine FF, regulates meiotic arrest via inhibition of oocyte PDE3 expression, increasing cGMP in the CC and cAMP in the oocyte, respectively. Estradiol also acts in the meiotic arrest by inhibiting PDE3 expression. However, current data did not show any effect on NPR2 mRNA expression.

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A060 Folliculogenesis, Oogenesis and Ovulation

The role of INOS/NO/CGMP pathway on *in vitro* maturation of bovine oocytes-cumulus complexes in presence of follicular wall hemisections

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Keywords: *in vitro* maturation, nitric oxide, nucleotides.

The role of nitric oxide (NO) in the mammalian oocyte maturation and the mechanisms involved in this process has not been completely understood. NO is produced by nitric oxide synthases (NOS) and stimulates the soluble guanylate cyclase enzyme (sGC) to produce cGMP. This nucleotide controls the action of phosphodiesterase 3A, which has the function of metabolizing cAMP to the inactive form, leading to the resumption of meiosis. Aiming to better understand these events, we used two active substances in this pathway: aminoguanidine (AG), which inhibits the inducible NOS isoform (iNOS); and 1H- [1,2,4] oxadiazol [4,3-a] quinoxalin-1-one (ODQ), which acts by inhibiting the sGC. Groups of 20 COC (120 COC/treatment) were cultured with eight follicular wall hemisections (HS) in an incubator at 38.5°C and 5% CO_2 in 200 µl of maturation medium (TCM-199/BSA) supplemented with AG (1 and 100 mM) and ODQ (10^{-3} M). The controls consisted of COCs cultured in the presence (C -) and absence of HS (C +). Oocyte nuclear maturation state was assessed by staining with 0.2% acetic orcein after 22h of IVM. The intracellular concentrations of cGMP (30 COC/treatment) and cAMP (10 COC/treatment and 50 oocytes/treatment) were measured at 0, 1, 3, 6, 9h for COCs and 0, 3, 6, 9h in the oocytes with an enzyme immunoassay. The results were analyzed with the Tukey test ($P<0.05$). The presence of HS (C -) decreased the percentage of oocytes that reached metaphase II (MII) (41.0 ± 4.0%) compared to the C + (78.5 ± 3.9%; $P <0.05$). Addition of 1 mM AG stimulated meiosis resumption compared to C - ($P<0.05$) and was the same as in C + ($P>0.05$), while 100 mM AG inhibited resumption of meiosis and progression to MII. The addition of ODQ stimulated meiosis resumption compared to C -. However, it inhibited the progression to MII (21.9 ± 3.5%; $P<0.05$). cGMP concentrations decreased over time in all experimental groups. The results observed in group with 1 mM AG do not differ from those observed in group C -. However, the addition of 100 mM AG and 10^{-3} M of ODQ decreased the concentration of cGMP, except at 3 and 6h. The group treated with ODQ had the lowest concentrations ($P<0.05$). The concentration of cAMP in COCs increased over time in all treatments ($P<0.05$). For this reason, this nucleotide was only measured in oocytes, where its concentration was descending. The C+ group had the lowest ($P<0.05$) concentration of cAMP, while groups treated with 100 mM AG and 10^{-3} M of ODQ had higher concentrations of cAMP ($P<0.05$). The results suggest that the activity of the iNOS/NO/cGMP pathway is important for maintaining the COC in the germinal vesicle stage (GV) of meiosis, and that progression to the MII is modulated by cGMP and cAMP concentrations in the COC/oocyte.

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A061 Folliculogenesis, Oogenesis and Ovulation

Protein profile of follicular fluid during folliculogenesis of the mare

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Keywords: follicular fluid, folliculogenesis, proteomics.

Follicular fluid (FF) is the only environment in which the avascular compartment of the follicle (granulosa cells and oocyte) are exposed and provides the microenvironment within which the cumulus-oocyte complex (COC) matures and granulosa cells differentiate. This experiment aims to compare the FF protein profile of the largest follicle (F1) and compare the largest and second largest follicle (F2) during different stages of follicular development (emergency, deviation, dominance and pre-ovulatory). Ovaries from 20 non-pregnant and cyclic mares were collected in an abattoir. Before slaughter, the mares were examined by transrectal palpation and ultrasound examination of the genital tract in order to evaluate ovaries and uterus. Blood samples were collected by jugular venipuncture. The diameter of the two largest follicles (F1 and F2) and the corpus luteum (CL) were obtained from each mare. Echotexture of the endometrium (EE) was evaluated and scored from 1 to 4. Mares were classified in the following experimental groups: G 15 (n = 4) F1 ≤ 15 mm, EE ≤ 2.5, CL > 27 mm; G 20 (n = 8) F1 20 to 26 mm, EE 2.5 to 3, CL 17 to 26 mm; G 30 (n = 4) F1 30 to 38 mm, EE > 3, CL < 16 mm; G 40 (n = 4) F1 > 40 mm, EE > 3, CL < 16 mm. Plasma progesterone concentrations were assayed by chemilluminescence. After slaughter, the FF of F1 and F2 was aspirated and submitted to 2D-PAGE for protein separation and identification by mass spectrometry. For statistical analysis a one-way analysis of variance (GLM procedure of SAS) was performed to evaluate the relative optical density of each protein spot as the dependent factor and the experimental groups, F1 and F2 and their interactions as independent variables. From the 20 mares studied, four constituted G15, eight G20, four G30 and four G40. Plasma progesterone concentrations varied from 8.1 to 12.7 in G15, 6.7 to 12.6 in G20, 0.6 to 1.3 in G30 and 0.6 to 0.7 in G40. A total of 43 spots was observed in gels (38 from F1 and 35 from F2). Nine spots presenting significant differences between treatments were submitted to mass spectrometry. Albumin, apolipoprotein A-I, gelsolin, serotransferrin and alpha-1-antiproteinase 2 were detected in the fluid of F1 and differed in abundance ($P < 0.05$) between the experimental groups. POM121 and ZP3 fusion protein (POMZP3) differed ($P = 0.02$) in abundance since deviation (G20), and alpha-1-antiproteinase 2 showed interaction ($P = 0.05$) between F1 and F2. The majority of the proteins identified in FF are present in blood plasma. It was not possible to correlate a specific protein with a particular stage of follicular development. However, serotransferrin and alpha-1-antiproteinase 2 had greater abundance during dominance and apolipoprotein A-1 and gelsolin during the pre-ovulatory stage. POMZP3 showed higher abundance in the dominant follicle compared to the subordinate one.

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A062 Folliculogenesis, Oogenesis and Ovulation

Blood perfusion in preovulatory follicle in Nelore cows under FTAI protocols

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Keywords: Doppler, estradiol benzoate, ovulation.

The objective of this study was to characterize changes in blood flow in the wall of the pre-ovulatory follicle during the latest wave of follicular growth since the withdrawal of progesterone implant until ovulation using an ovulation-inducing protocol in Nelore cows. Twenty eight cows were divided randomly into two protocols, the protocol BED9 (n=14) consisted of placing an intravaginal progesterone implant (Sincrogest®, Ourofino, Cravinhos-SP) and 2mg IM estradiol benzoate (BE, Benzoato HC, Hertape Calier, Juatuba-MG) in D0; withdrawal of progesterone implant and 0.150mg of PGF2α (Veteglan®, Hertape Calier, Juatuba-MG) IM in D8 and 1mg of BE IM after 24 h. The protocol BED8 (n=14) consisted of placing an intravaginal progesterone implant and 2mg IM estradiol benzoate on D0; withdrawal of progesterone implant, injection of 0.150mg de PGF2α and 1mg of BE in D8, in both cases IM. Animals were evaluated by color doppler ultrasound (MyLab™30Gold, Esaote) every 6 hours for 90 hours or until ovulation of the pre ovulatory follicle. Hour zero corresponded to the time of progesterone implant removal. Of the 28 cows, 11 (39.3%) did not ovulate before 90 hours, 4 of BED9 and 7 of BED8. Follicle vascularization was subjectively assessed using a score system in which follicles with from 0 to 20% of its circumference irrigated were classified as grade 1, from 20 to 40% as grade 2, from 40 to 60% as grade 3, from 60 to 80% as grade 4 and from 80 to 100% as grade 5. For statistical analyzes of the ovulation time and size of the pre-ovulatory follicle, t-tests were used for normal distribution and the Mann-Whitney test for non-normal distribution ($P < 0.05$). For the total number of follicles in each time evaluated ranked at different grades of irrigation the Fisher's test was used ($p < 0.05$). The average size of the ovulated follicles was 12.83 ± 1.31 and 11.85 ± 1.71mm ($p = 0.20$) in BED9 and BED8, respectively. A statistical difference was observed for the time of of ovulation; 74.4 ± 3.9 in BED9 and 61.71±11.33 hours in BED8 ($p = 0.01$). Regarding the vasculature, it was observed that in BED9 the first 24 h most of the follicles remained at grade 2, in the range from 24 to 30 h it was observed a change of vascularization from grade 2 to 3, grade 3 to 4 in the range from 36 to 42 hours and grade 4 to grade 5 in the range from 66 to 72 hours. In BED8, it was observed that from 0 to 30 hours, most follicles remained at grade 3, from 30 to 36 h there was an increase in the percentage of follicles at grade 4, and from 54 to 60 hours an increase of follicles at grade 4 to 5. The results suggest that the use of BE at the moment of P4 withdrawal hastens ovulation compared to injection 24 h later, and that near ovulation there is an increase in the vascularization of the wall of the pre-ovulatory follicle, in which Color Doppler can distinguish the pre-ovulatory follicle by its irrigation and ovulation proximity.

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A063 Folliculogenesis, Oogenesis and Ovulation

Regulation of TGFβ family members around follicular deviation and final growth in bovine

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Keywords: follicular growth, GDF9, growth factors.

The family members of the transforming growth factor β (TGFβ) have been studied since the description of the naturally occurring BMP15 and GDF9 mutations in sheep, which are associated with infertility in homozygous or increased ovulation rate in heterozygous. However, the regulation and function of TGFβ family members around follicular deviation in bovine have not been completely characterized. Therefore, the objective of this study was to investigate the expression of inhibin (Alpha, BetaA and BetaB) and BMPs (1, 2 and 4) during follicular deviation, and the effect of intrafollicular injection (IFI) of GDF9 on growth of dominant follicles in cattle. Ovaries were collected by colpotomy and granulosa cells were isolated from the two largest follicles before (day 2; F1 and F2; n=4), during (day 3; dominant follicle (DF) and subordinate (SF); n=4) or after (day 4; DF and SF; n=6) deviation (Evans and Fortune, 1997. Endocrinology, v.138, p.2963–2971). In order to investigate the effect of GDF9 on follicular growth, cows were synchronized (n=11) and IFI of either vehicle (PBS; control group, n=5) or 100 ng/mL recombinant human GDF9 (GDF9) as final intrafollicular concentration (n=6; starting dilution of 1000 ng/mL) was performed when the DF reached 9 to 10mm. Follicular growth was monitored every 24h after treatment. All data were tested for normal distribution using Shapiro-Wilk test, normalized by log transformation when necessary and submitted to ANOVA. The effect of GDF9 on follicular development was evaluated as repeated measures data using the MIXED procedure. The deviation model was validated by assessing the transcript levels of CYP19A1, which was higher in the DF ($P<0.05$) during and after deviation. Moreover, mRNA levels of inhibin Alpha (INHA), BetaA (INHBA) and BetaB (INHBB) were consistently higher in the DF. INHBB mRNA was more abundant before (day 2), during (day 3) and after (day 4), while INHBA transcripts were higher during and after dominance. INHA mRNA levels were higher in the DF after follicular dominance. The relative levels of BMP1 mRNA were significantly higher ($P<0.05$) in F2 on day 2 of the follicular wave, but similar between DF and SF on days 3 and 4. BMP2 mRNA abundance did not differ between the two largest follicles, but BMP4 mRNA was more abundant in the DF on days 3 and 4. The IFI of GDF9 did not affect follicular growth and ovulation, as all the cows in the control group (n=5) and five out of six cows in the GDF9 group ovulated three to four days after treatment. These results revealed that BMP1 is expressed in the bovine ovary, and its expression pattern suggests a potential inhibitory role on the growth of the future SF. Transcript levels suggest that BMP2 is likely not involved in the regulation of follicular deviation while BMP4 may be associated with follicular dominance. Finally, GDF9 seems to not alter the development of large follicles and ovulation, however, other concentrations of the peptide should be tested.

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A064 Folliculogenesis, Oogenesis and Ovulation

Resveratrol mantains survival and promotes cell proliferation in ovine preantral follicles cultured *in situ*

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Keywords: antioxidant, oocyte, sheep.

Some studies have demonstrated that resveratrol, a fitoalexin produced by several plants and present in grapes, reduces follicular atresia (KONG et al., J Endocrinol Invest., v. 34, p. 374-81, 2011) and reactive oxygen species (KWAK, et al., Theriogenology, v. 78, p. 86-101, 2012) after rat ovarian follicle culture. However, there are no reports about the effect of resveratrol on the in vitro culture of ovine ovarian preantral follicles. Thus, the aim of this study is to evaluate the effect of resveratrol on the morphology, activation and cell proliferation of ovine preantral follicles cultured in situ. After collection of ovine ovaries (n=10) in the slaughterhouse, ovarian cortex was fragmented and one fragment was immediately fixed and destined to histology (fresh control). The remaining fragments were cultured in vitro for 7 days in α-Minimal Essential Medium (α-MEM – GIBCO, Invitrogen, St Louis, EUA) supplemented with insulin, transferrin, selenium, hypoxanthine, glutamine, ascorbic acid and BSA (α-MEM+ - control medium) or in α-MEM+ added by different concentrations of resveratrol (2; 10 or 30 μM). After culture, preantral follicles were morphologically analysed and classified as normal or atretic according to the absence or presence of cytoplasmic shrinkage, nuclear pycnosis and/or disorganization of granulosa cells, as well as classified as primordial or growing follicles (intermediate, primary and secondary follicles). Immunohistochemical analysis was also perfomed for detection of Proliferation Cell Nuclear Antigen (PCNA; 1:500; Santa Cruz Biotechnology; Santa Cruz, CA, EUA). The PCNA positive cells (brown) were counted in the sections and expressed as percentage. The percentage of normal, primordial and growing follicles were compared by ANOVA and Tukey's test ($P<0.05$). After 7 days of culture, there was a significant reduction in the percentage of morphologically normal follicles compared to the fresh control. The concentration of 2 μM resveratrol showed percentages of normal follicles similar to the control medium and significantly higher than other resveratrol concentrations. In addition, there was a significant reduction in the percentage of primordial follicles and an increase in the percentage of growing follicles (follicular activation) in all treatments compared to the fresh control. Moreover, it was possible to observe a significant increase in the percentage of PCNA positive cells in follicles cultured in 2 μM resveratrol (50.7%) in comparison to fresh control (34.0%) and α-MEM+ (4.3%). In conclusion, resveratrol at 2μM maintains survival, promotes activation and cell proliferation of ovine preantral follicles cultured in situ.

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A065 Folliculogenesis, Oogenesis and Ovulation

Omega 3 supplementation to prepubertal gilts increases leptin and its receptor in preantral follicles

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Keywords: gilts, leptin, Omega 3.

The main approach to anticipate puberty is providing high energy diet to gilts to reach satisfactory body condition score (AMARAL; Animal Reproduction Science; 121:139-144, 2010). Diets containing high levels of omega 3 fatty acids increase serum leptin regulating its synthesis and secretion. The objectives of the present study were to evaluate body weight and backfat depth in prepubertal gilts supplemented with omega 3 source as well as the effect of omega 3 supplementation on reproductive organs. Prepubertal gilts were daily supplemented with 9 mL soybean oil (control group; n=13) or 9 mL fish oil with 5 g omega 3 containing 33% DHA and 22% EPA (omega 3 group; n=12) during 45 days. After slaughter, uteri were weighed and ovaries evaluated to verify the presence of corpus luteum (CL). For immunohistochemistry (IHC), ovarian sections (n=4/group) were incubated with primary polyclonal antibodies anti-leptin (Ob; 1:2000) and anti-leptin receptor (ObRb; 1:100). Oocytes were classified as included in primordial/primary follicles (OIPF); secondary follicles (OISF) or tertiary follicles (OITF). Protein quantification was done by software image analysis (ImageJ® software) to obtain the most common value (the mode) for each area (MOREIRA; Animal Reproduction Science; 139: 89-94, 2013). Analysis of productive parameters was performed as repeated measures data and analyzed using the MIXED procedure (SAS®). ANOVA was used to test for effects of omega 3 on uterus weight and immunostaining intensity and differences between means were determined with Tukey test using Statistix® software (2008). Body weight did not differ ($P>0.05$) between groups at any time. However, a significant interaction between group and moment ($P<0.05$) was observed, being observed higher backfat deposition in omega 3 gilts 45 days after the beginning of supplementation ($P=0.06$). CLs were not detected whereas uterus weight tended to be higher in omega 3 gilts ($P=0.09$) after slaughter. In omega 3 gilts, immunostaining for leptin was higher in OIPF and OISF ($P<0.05$) compared to control gilts, but no differences were observed in OITF ($P>0.05$). For leptin receptor, it was observed higher immunostaining ($P<0.05$) in OISF from omega 3 gilts and no differences were observed in other follicular phases ($P>0.05$). Collectively, data suggest that omega 3 supplementation increases the chance of reaching satisfactory body condition score at puberty and that increased leptin levels induced through omega 3 supplementation may positively influence oocyte/follicle and reproductive tract development.

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A066 Folliculogenesis, Oogenesis and Ovulation

Validation of molecular markers for oocyte competence in bovine cumulus cells

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Keywords: individual culture, markers, oocyte competence.

Considering that cumulus cells (CC) have a bi-directional communication with the oocyte and that they play an important role in the growth and maturation, they can be used to indicate oocyte quality in a noninvasive way. Several studies have identified candidate genes in CCs, which expression is associated with oocyte competence and can be used as markers. However, to prove the efficiency of those as markers, it is necessary to follow the development of each cumulus-oocyte-complex (COC) individually until the blastocyst formation. The present study aimed to quantify the expression of candidate genes in CC from COC with high and low potential to develop in vitro up to the blastocyst stage. The different culture systems were used for IMV, IVF and IVC. Initially, the effect of the individual culture system and biopsy on embryo development was evaluated and COCs were distributed into 3 groups: control (COCs were cultured in groups); WOW (COCs were cultured individually in the WOW system); and micro droplet (COCs were individually cultured in micro droplet of 20 μL). Then, embryo production was compared between the control and the individual system (micro droplet), in which the COCs were submitted or not to biopsy. Expression levels of GPC4, IGFBP4, FSHR, GHR, EGFR, FGF11, SLC2A1, SLC2A3, SPRY1, VCAN and KRT8 genes were quantified by real time PCR (RT-qPCR) in 5 pools with 7 CC biopsies obtained from immature COC. Each biopsied COC was individually tracked by culturing them in a micro droplet, and categorized based on his fate: embryo at expanded blastocyst stage at D7, cleaved and arrested and not cleaved. Blastocyst rates were lower in individual culture systems (WOW = 17.9% n=95; microdrop = 26.3% n=95) than in the control group (45.0 %, n=209). However, no effect of the biopsy was observed for both groups (P>0.05). From the 11 genes evaluated, 3 showed differential expression. Higher expression of GHR (P=0.09) and VCAN (P=0.06) was observed in CCs that formed embryos compared to those that did not cleave. The GPC4 gene was overexpressed (P=0.007) in CC from formed embryos compared to cleaved and arrested ones. It was concluded that individual culture reduced blastocyst production, but biopsy did not affect embryo development. The expression of GHR, VCAN and GPC4 genes can be used as markers to distinguish COCs associated with embryo development from COCs with limited developmental potential.

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A067E Folliculogenesis, Oogenesis and Ovulation

Osmotic challenge of bovine early pre-antral follicles with different cryoprotectant agents

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Keywords: bovine preantral follicle, cryoprotectant agent, DMSO, EG, follicle permeability, osmotic challenge.

Significant advances in cancer diagnosis and treatments have stimulated interest in fertility preservation strategies as chemical or ionizing radiotherapy often threatens future reproduction. Ovarian tissue cryopreservation currently is the only option for preserving the reproduction potential of pre-pubertal girls and women whom cannot undergo hormonal stimulation, ovarian tissue cryopreservation currently is the only option. However, there is a huge concern regarding the possible presence of malignant cells in the retrieved ovarian tissue, which could lead to cancer reintroduction after reimplantation of the frozen-thawed tissue strip. Cryopreservation of isolated early preantral follicles (PAFs) (and subsequent *in vitro* culture (IVC), maturation (IVM) and fertilisation (IVF)) might therefore represent an interesting alternative. Existing protocols are based on protocols for freezing embryos and oocytes . However, in order to improve follicular survival after cryopreservation, it is essential to develop a protocol for follicles specifically. Indeed, follicles are quite different from both embryos and oocytes, if only because they are composed of two different cell types (namely the oocyte and the surrounding (pre-)granulosa cells). In order to provide a biophysical base for choosing optimal cryoprotectant agents (CPAs) that avoid severe volume changes and formation of intracellular ice crystals, in this experiment, two-day-old isolated bovine PAFs were osmotically challenged by exposing them to different concentrations of cryoprotectant agents (CPAs). Briefly, isolated bovine early PAFs were exposed to either ethylene glycol (EG) or dimethyl sulfoxide (DMSO) in different final concentrations: 1 M, 2 M, 3 M, 4 M and 5 M at room temperature, and photographed at different time points (every half minute between 0 and 5 minutes) after the onset of exposure to calculate their volume over time (5 - 10 follicles per CPA and per concentration). Although there was a high variability in the individual response of the follicles to this CPA challenge, all follicles showed a typical ‘shrink/swell’ curve. Analysis with two-way ANOVA showed no interaction between the type of CPA and the respective concentrations. This means that volume differences in time between the minimum and maximum for both EG and DMSO were uniform across concentrations. Across all concentrations, time until the post stimulus maximum (i.e. the maximum volume to which follicles re-expand after shrinkage) appeared significantly longer in the EG group ($P = 0.04$), indicating that bovine early PAFs are less permeable to EG than DMSO. To our knowledge, this is the first time that isolated bovine early PAFs were osmotically challenged by exposing them to different concentrations of penetrating CPAs. This has provided us with some basic insights in follicular permeability to CPAs. These insights are a first step in the design of cryopreservation protocols for isolated early PAFs specifically.

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A068E Folliculogenesis, Oogenesis and Ovulation

Developmental competence of porcine oocytes that have finished growth phase from follicles of different diameter

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Keywords: Brilliant cresyl blue, diameter, follicular development, pig oocytes.

Numerous factors determined developmental competence of the oocytes. Brilliant cresyl blue (BCB) staining has been used for selection of oocytes from several mammalian species, including pigs (Ericsson S. et al, 1993 Theriogenology, 39(1): p.214). The aim of the present study was to evaluate the developmental competence of porcine oocytes that have finished growth phase (BCB^+) depending on diameter (d) of follicles (d < 3 mm, 3 - 6 mm, >6 mm) and to detect expression of estrogen receptor (ER) in cumulus cells of BCB^+ and BCB^- oocytes. Before IVM compact cumulus oocyte complexes (COC) were incubated in BCB solution for 60 minutes. Treated oocytes were divided into BCB^- (colorless cytoplasm) and BCB^+ (colored cytoplasm). Only BCB^+ oocytes were used in the experiments. The medium used for oocyte maturation was NCSU 23 supplemented with 10% follicle fluid (FF), 0.1 mg/ml cysteine,10 IU/ml eCG and 10 IU/ml hCG. FF was collected from follicles with 3 - 6 mm in diameter, COC cultured in maturation medium with pieces of wall (600-900 μm in length) from non atretic healthy follicles (d 3-6 mm). After 20-22 h of culture, COC and pieces of wall were washed and transferred into the same maturation medium but without hormonal supplements for another 20-22 h of culture. After IVM oocytes were fertilized in vitro and embryos were cultured by standard protocols (Stokes P. et al., Developmental Biology, 284, p.62 – 71, 2005). All chemicals used in this study were purchased from Sigma-Aldrich (Moscow, Russia). The question was: have BCB^+ oocytes from follicles of different diameters the same developmental competence? We did not find significant differences between the level of cleavage and blastocyst in all groups of experiments. Percentages of cleavage and blastocyst in groups were: follicles d < 3 mm - 43% (27/63) and 29% (18/63); follicles d 3 - 6 mm - 46% (45/98) and 35% (34/98); follicles d > 6 - 48% (28/58) and 28% (16/58) (χ^2 test). Immunocytochemical analysis was used for detection of *estrogen receptor* expression (ER) in cumulus cells of 53 BCB^+ and 33 BCB^- oocytes. Immunocytochemical staining was performed using the first rabbit polyclonal anti-human ER antibodies (NCL-ERp, Novocastra, OOO BMS, St.Petersburg, Russia). The visualization system (ABC-universal kit) consists of avidin–biotinilated peroxidase (DakoCytomation) was applied. 3,3'-diaminobenzidine was used as it was recommended from manufacture Novocastra (OOO BMS, St.Petersburg, Russia). Hematoxylin *(Abrisplus,* St.Petersburg, Russia) was used to stain cells. Antigen optical density was measured using morphometric VideoTest (Russia) computer program. Positive immunocytochemical reaction was mainly observed in the nuclei membrane and slightly on the cytoplasmic membrane of cumulus cells (probably as non-specific background). It was shown that cumulus cells of BCB^+ oocytes had ~~a~~ significantly more pronounced expression of the ER than the cumulus cells of BCB^- oocytes ($p < 0,001$, Mann-Whitney test).

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A069E Folliculogenesis, Oogenesis and Ovulation

Determining intrafollicular concentrations of cortisol and progesterone in horses and the effects of cortisol on *in vitro* maturation of equine oocytes

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Keywords: cortisol, follicles, horse, progesterone, IVM, oocytes.

Increased glucocorticoid release and synthesis in response to acute or chronic stress has been shown to impair reproductive function in a variety of species and therefore may affect fertility. The aims of this study were 1) to determine cortisol and progesterone concentrations in equine follicular fluid and serum and 2) to assess the effects of cortisone supplementation to the maturation medium on IVM rates of equine oocytes. We hypothesized that challenging equine oocytes during IVM with higher doses of cortisone than physiological levels does not affect IVM rates. Light horse mares (n=9) used in this study were reproductively sound and cycling. Follicular fluid samples were collected by ultrasound-guided transvaginal follicle aspiration from the following follicle classes: G1: 5-9 mm, G2: 10-14 mm, G3: 15-19 mm, G4: 20-24 mm and G5≥25 mm. Blood samples were collected from each animal at the beginning and at the end of the aspiration period, respectively. Hormone determinations for cortisol (DE1887, Demeditec, Kiel-Wellsee, Germany) and for progesterone (ADI-901-011, Enzo Life Sciences, Farmingdale, NY, USA) were performed by ELISA. Cumulus oocyte complexes (COCs) were collected by OPU only from healthy, growing follicles, in the absence of a preovulatory follicle. Compact COCs (n=84) were randomly assessed either to control group, or to one of the treatment groups, in which hydrocortisone (H4001, Sigma Chemical, St. Louis, MO, USA) was added to the standard maturation medium in the following concentrations: 0.1 µg/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml. After 30h, oocytes were denuded, stained with Hoechst (33342, Sigma) and IVM rates were assessed. Statistical analysis was done with the SPSS Statistics 22 software. As all data were normally distributed (Kolmogorov–Smirnov test, $p > 0.05$ for all parameters), one way ANOVA, Post-Hoc-Test and Pearson's correlation were applied for the hormones, whereas Chi-Square Test was used to analyse IVM rates. In follicular fluid from G5 follicles, concentrations of cortisol and progesterone were significantly higher ($p<0.05$) than in all other groups. Concentrations of cortisol and progesterone were positively correlated ($r=0.8$; $p<0.001$). In contrast, serum concentrations of progesterone and cortisol in mares did not differ at the beginning and the end of the aspiration period. There was no significant difference in the percentage of matured oocytes between groups, regardless of the concentration of cortisone added to the medium. Our results demonstrate a significant increase of cortisol in preovulatory follicles in vivo, suggesting its importance for oocyte maturation. Moreover, challenging equine oocytes in vitro with up to 100 times more cortisol than physiologically existent in follicles larger than 25 mm did not significantly affect IVM rates, suggesting that the equine oocyte is able to modulate cortisol levels and therefore to adapt to stress situation.

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A070 FTAI, FTET and AI

Second TAI with an early resynchronization in nulliparous and mutiparous Nelore females

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Keywords: early resynchronization, Nelore, TAI.

The aim of this trial was to compare the use of an early resynchronization (before pregnancy diagnosis) in Nulliparous and Multiparous Nelore females. A total of 308 Nulliparous and 142 Multiparous Nelore cows, with an average BCS of 2.84 ± 0.01 were used. In the first TAI protocol the animals were synchronized with the following protocol: insert of an intravaginal progesterone device (CIDR 1.9g; Zoetis, Sao Paulo, Brazil) and 2.0 mg im of estradiol benzoate (EB; Gonadiol, Zoetis) on Day -11, 12.5 mg im of dinoprost tromethamine (PGF; Lutalyse, Zoetis) on Day -4, on Day -2 CIDR was removed and 0.5 mg im of estradiol cypionate (ECP; Zoetis) and eCG (200 heifers or 300 cows; Novormon; Zoetis) were administered. The TAI was performed on Day 0, 48 h after CIDR withdrawal. On Day 23, all animals received the insertion of a CIDR and 1.0 mg im of EB. On Day 30, the pregnancy diagnosis was performed and the CIDR was removed. The nonpregnant females also received 12.5 mg im of PGF. On Day 32 CIDR was removed and were administered 0.5 mg im of ECP and eCG (200 Nulliparous or 300 Multiparous). The second TAI was performed on Day 34, 48 h after CIDR removal. Pregnancy rates to 1st and 2nd TAI were analyzed using PROC GLIMMIX from SAS, being included in the models the effects of category, BCS, AI tech and AI sire. For analysis of the final pregnancy rate (1st+2nd TAIs) was included in the model the effect of category. Results were reported as least square means. None of the variables tested were significant in pregnancy rates for 1st and 2nd TAI. For Nulliparous pregnancy rates was 63.6% (211/308) and 55.7% (54/97) for 1st and 2nd TAI respectively. Multiparous cows pregnancy rates was 54.1% (68/142) and 63.5% (47/74) for 1st and 2nd TAI, respectively. The final pregnancy rate did not differ between categories, being 86.0% (265/308) and 80.9% (115/142) for Nulliparous and Multiparous Nellore females, respectively. The utilization of a 2nd TAI protocol using early resynchronization, before pregnancy diagnosis, did not differ between both, Nulliparous and Multiparous Nelore females, indicating be a viable alternative to improve the pregnancy rate early in the breeding season.

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A071 FTAI, FTET and AI

The addition of GnRH and/or anticipated PGF2α in progestogen/estradiol TAI-based protocol does not affect dairy cows fertility

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Keywords: lactating cow, pregnancy rate, synchronization.

The aim of the present study was to evaluate the reproductive performance of lactating dairy cows submitted to different synchronization protocols for TAI. A total of 422 lactating dairy cows (Holstein and Holstein x Jersey), reared in loose-housing system with milk yield of 23.4 ± 0.4 L/cow/day, body condition score (BCS) 3.0 ± 0.02 (range 1-5) and 144.8 ± 4.5 days in milk (DIM) from five commercial dairy farms was enrolled in the experiment. Cows were homogeneously distributed in four groups (control, n = 102; GnRHD0, n = 109; PGFD7, n = 102 and; GnRHD0 + PGFD7, n = 109) in a factorial 2 x 2 design considering milk yield, BCS and DIM. On a random day of the estrous cycle all animals were submitted to a synchronization protocol for TAI [Day 0: 2 mg of estradiol benzoate (EB; Gonadiol®, MSD, Argentina) + norgestomet ear implant (Crestar®, MSD, Netherlands); Day 8: implant removal + 0.530 mg of sodium cloprostenol (PGF2α; Ciosin®, MSD, Brazil); Day 9: 1 mg EB (Gonadiol®, MSD, Argentina); Day 10 AM: TAI]. In addition to the described protocol, the GnRHD0 group received 100 µg of gonadorelin (Fertagyl®, MSD, Netherlands) on Day 0, the group of PGFD7, 0.530 mg of sodium cloprostenol (Ciosin®, MSD, Brazil) on Day 7 and the GnRHD0+PGFD7 group, 100µg of gonadorelin on Day 0 and 0.530 mg of sodium cloprostenol on Day 7. The animals in the Control group received no additional treatment. On Day 40, females were evaluated to pregnancy diagnosis by ultrasound exam. Statistical analysis was performed by GLIMMIX the SAS®. No interaction was observed between treatment with GnRH on Day 0 and treatment with PGF2α on Day 7 for pregnancy rate 30 days after TAI [Control: 40.2 (41/102); GnRHD0: 37.6 (49/109); PGFD7: 38.2 (39/102) and; GnRHD0+PGFD7: 42.2% (46/109); P = 0.47]. Also, no effect of treatment with GnRH on Day 0 [With GnRHD0: 39.9 (87/218) and No GnRHD0: 39.2% (80/204); P = 0.82] or PGF2α on Day 7 [With PGFD7: 40.3 (85/211) and No PGFD7: 38.9% (82/211); P = 0.52] was verified. Additionally, although it was observed farm effect (P = 0.03), no interaction farm and treatment (P > 0.05) was established. Therefore, the addition of GnRH at the beginning of the protocol and/or PGF2α on the day before of ear implant removal do not increase pregnancy rate in dairy cows (Holstein and Holstein x Jersey) reared in loose-housing system submitted to progestogen/estradiol TAI based protocol.

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A072 FTAI, FTET and AI

Does the use of eCG in TAI protocols improves follicular and luteal function of pregnant and non pregnant crossbred dairy cows?

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Keywords: corpus luteum, follicular diameter, progesterone.

This study aimed to determine the impact of the use of equine corionic gonadotropin (eCG) on follicular diameter (DFOL), luteal (DCL) and concentration of progesterone (P4) in pregnant and non pregnant cows submitted to a synchronization protocol for timed AI (TAI). A total of 126 crossbred dairy cows were subjected to the following protocol: on a random day of the estrous cycle (Day 0) was applied an intravaginal progesterone device (CIDR®, Zoetis, São Paulo, Brazil) associated with 2 mg of estradiol benzoate IM (Gonadiol®, Zoetis). At Day 8 the P4 device was removed and were administered 25 mg of dinoprost trometamina (Lutalyse®, Zoetis) and 1 mg of estradiol cypionate (E.C.P.®, Zoetis). At this time, cows were randomly assigned to one of two treatments: eCG (eCG; n = 61) 300IU of eCG IM (NOVORMON®, Zoetis, São Paulo, Brazil) was administred and No eCG (n = 65) without treatment. On Day 10, immedially before the TAI, cows had their DFOL meansured by transrectal ultrasonography. On Day 20, the DCL was meansured and blood collected for P4 concentration determination using the chemiluminescence. Pregnancy diagnosis was perfomed 30 days after TAI by ultrasound. Thus, four groups were established: eCG pregnant (n = 25), eCG not pregnant (n = 30), No eCG pregnant (n = 21) and No eCG not pregnant (n = 37). Statistical analysis was performed using SPSS (version 19) program, considering 5% significance. The DFOL were similar among groups (eCG pregnant, eCG non-pregnant, No eCG pregnant and No eCG nonpregnant: 10.1±3.4; 11.2±3.1; 12.1±2.4 and 11.1±2.9mm, respectively). The DCL were respectively; 19.8±3.8; 21.4±4.2; 20.5±3.7 and 19.9±3.8mm, also did not differ. However, the eCG pregnant group showed significant difference (P = 0.0007) for P4 concentration (16.7±11.1 ng/mL) compared with other groups eCG nonpregnant (8.2±6.7 ng/mL), No eCG pregnant (8.2±5.7 ng/mL) and No eCG nonpregnant (8.7±6.7 ng/mL), these last three did not differ significantly. In conclusion, pregnant cows treated with eCG had better luteal function and consequently higher concentration of P4.

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A073 FTAI, FTET and AI

Addition of a second dose of prostaglandin F2α to a fixed-time AI protocol improves fertility of anestrous dairy cows and without hyperthermia

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Keywords: anestrus, PGF2α, TAI.

This study was conducted during January to December 2014 in two dairy farms in the Minas Gerais State - Brazil, the aim was to evaluate if addition of a second prostaglandin (PG) F2α dose improves fertility of anestrous lactating Holstein cows submitted to an estradiol/progesrterone(P4) based timed AI (TAI) protocol. Ovaries were scanned by ultrasound to determine if a corpus luteum (CL) was present at the protocol initiation (d-11) and on the day of PGF2α (d-4). Cows without CL on d-11 and d-4 were classified as anestrous (n=436). Anestrous cows were submitted to the TAI protocol: d-11: two intravaginal P4 devices (1.9g P4,CIDR, Zoetis) and 2.0 mg of estradiol benzoate (EB) i.m. (Gonadiol, Zoetis); d-4, 25mg of PGF2α i.m. (Lutalyse, Zoetis) and withdrawal of one CIDR; d-2 withdrawal of the second CIDR and 1.0 mg i.m. of estradiol cypionate (E.C.P., Zoetis); on d0 TAI. On d-4, cows were randomly assigned to one of two treatments: one dose of PGF2α on d-4 (1PGF) or two doses of PGF2α (2PGF), the first on d -4 and the second on d-2. Rectal temperature was measured on the day of TAI and 7 days later. The average rectal temperature was calculated and the cows were divided into two groups: without hyperthermia (<39.1°C) or with hyperthermia (≥39.1°C). Pregnancy was diagnosed 60 d after AI. Binomial variables were analyzed using the GLIMMIX and continuous using the MIXED procedures of SAS. The synchronization rate in this study was 76.4% (n = 436) and the percentage of cows with hyperthermia was 58% (n = 436). The 2PGF treatment tended to improve the synchronization rate (80.2% [n=218] vs. 72.4% [n=218] P=0.07). An interaction (P=0.05) between treatment and hyperthermia was observed for pregnancy per AI (P/AI) on d 60. For hyperthermic cows, P/AI were 9.1% (n=123) and 9.4% (n=130) for 1PGF and 2PGF, respectively. For cows without hyperthermia, P/AI were 16.3% (n=95) and 30.0% (n=88) in 1PGF and 2PGF, respectively. When only ovulated cows were included in the analysis, then an interaction (P=0.05) existed between treatment and hyperthermia on P/AI at 60d. For cows with hyperthermia, P/AI were 13.8% (n=80) and 12.1% (n=100) in 1PGF and 2PGF, respectively. For cows not suffering of hyperthermia, P/AI were 19.4% (n=78) and 34.8% (n=75) in 1PGF and 2PGF, respectively. The addition of a second PGF2α dose during the TAI protocol increased fertility in anestrous dairy cows without hyperthermia on the first 7 days after AI.

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A074 FTAI, FTET and AI

Adjustment of the estradiol benzoate dose in the resynchronization protocol with unknown pregnancy status in suckled beef cows

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Keywords: bovine, fertility, pregnancy loss.

This research aimed evaluated two doses of estradiol benzoate (BE) administered early in the resynchronization protocol 22 days (Day 22) after the first timed-AI (TAI) on the pregnancy rate by TAI (P/AI), pregnancy loss (PL) and induction of new follicular wave emergence (FW) in suckled beef cows. After 1st TAI and regardless of pregnancy status, 1426 cows (768 *B. taurus* and 728 *B. indicus*) were randomly divided into 2 groups according to the dose of BE [1 mg (n = 721) or 2 mg (n = 705); Bos taurus (RIC-BE®) and zebu (Gonadiol®)]. On day 22, cows received an intravaginal progesterone device (P4; PRIMER® and DIB®, taurine and zebu respectively). After 8 days (Day 30), the P4 removed and pregnancy diagnosis accomplished by ultrasound. The non-pregnant females received prostaglandin analogue [Bos taurus (Estron®) or zebu (Ciosin®)] IM. The Bos taurus cows received 10 mg of FSH (Foltropin®) and 1 mg of EB, whereas Zebu were treated with 300 IU eCG (Novormon®) and 1 mg estradiol cypionate (ECP®). The TAI was performed 44h or 48h after removal of the P4, Zebu and Bos taurus cows, respectively. Pregnancy diagnosis conducted at 30 and 62 days after 1st TAI. The data submitted for analysis with GLIMMIX (SAS 9.3). There was no interaction between treatments and breeds on the P/AI after the 1st TAI (P = 0.85), P/AI after 2nd TAI (P = 0.31), PL between 30 and 62 days after 1st TAI (P = 0.50), as well as the cumulative pregnancy (1st TAI+2nd TAI, P = 0.75). The P/AI after 1st TAI was similar (P = 0.85) among treatments (1 mg and 2 mg = 44.0%). However, the ratio P/AI after resynchronization was higher (P = 0.0001) in cows treated with 2 mg (1 mg = 36.1% and 2 mg = 47.3%). The PL at 1st TAI was similar (P = 0.37) among treatments (1 mg=3.8% and 2 mg=5.5%), however, the cumulative pregnancy was higher (P = 0.01) in cows treated with 2 mg of EB (68.2%) than those treated with 1 mg of EB (62.8%). A total of 40 zebu cows had their ovarian dynamics evaluated by ultrasound to assess the induction of a new follicular wave emergence after the treatment with different doses (1mg vs. 2mg) of BE 22 days after 1st TAI. In non-pregnant cows (1mg n= 12 and 2mg n= 12), despite of similar interval from EB treatment to new follicular wave emergence (mean ± SEM; P = 0.13), the emergence of new wave was more dispersed (P = 0.03) in those cows treated with 1mg of EB (1.8 ± 1.3 days) compared to cows treated with 2mg of BE (2.3 ± 0.6 days). Therefore, the dose of 2mg of BE is more efficient to induce a synchronous new follicular wave emergence, greater pregnancy per AI after resynchronization, without compromise the pregnancy established from the 1st TAI in suckled beef cows resynchronized with unknown pregnancy status.

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A075 FTAI, FTET and AI

Association between pin-bone angle, calving difficulty and fertility in lactating Holstein cows

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Keywords: dairy cow, fertility, pin-bone angle.

The objective of this study was to assess the possible association between pin-bone angle and fertility in lactating Holstein cows. Pin-bone angle was measured in 557 Holstein cows producing 38.2 ± 2.8 kg/dia and at 151 ± 12 DIM, housed in two free-stall herds in USA (herd 1 in California, herd 2 in Wisconsin). Pin-bode angle was with a digital angle-meter (ADA Angle-meter®) by a single technician without previous knowledge of the reproductive history of the cows. Data regarding calving difficulty (CD; scale of 1 ease to 3 very difficult) and conception results to 1st post-partum AI (CR1) were retrieved from the herd's software (Dairy Comp-305), used in both studied herds. Information of pin-bone angle was divided into quartiles, as follows: Q1 = cows with pin-bone angle of -2.0 to 0.2; Q2 = 0.3 to 1.0; Q3 = 1.1 to 1.7; Q4 = 1.8 to 5.3. Data was analyzed with the proc Glimmix of SAS (Version 9.3). There was no herd effect on CR1 (herd 1 = 27.9%; herd 2 = 32.3%; P = 0.26); however, herd 2 had a greater proportion of cows having a difficult calving CD > 1 (herd 1 = 8.0%; herd 2 = 19.7%; P = 0.03). Parity tended to affect CD (P=0.10) and CR1 (P=0.09), and calving month had no effect (P>0.10) on CD or CR1. Unexpectedly, pin-bone angle did not influence any studied variables (CD: P = 0.53; CR1: P = 0.68). Thus, the average CD and CR1 for each quartile was, respectively: Q1 = 1.2 and 33.5%; Q2 = 1.2 and 26.5%; Q3 = 1.2 and 25.6%; Q4 = 1.3 and 29.8%. In contrast to our initial hypothesis, these results suggest little to no influence of the pin-bone angle on calving difficulty and conception results to 1st postpartum AI in high producing Holstein cows.

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A076 FTAI, FTET and AI

Delayed insemination time in taurine heifers that did not display estrus after progesterone device removal

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Keywords: GnRH, heat, heifers.

Occurrence of estrus after synchronization of ovulation protocol is associated with a greater pregnancy rate following timed artificial insemination (TAI) protocols. However females that do not display estrus, present delayed ovulation (Lopes, M.S.B., et al., Anim. reproduction, v.9, n.3, p.520, 2012.) and low pregnancy per AI (P/AI) (Sá Filho et al. Theriogenology, v. 76, p. 455-63, 2011). Thus, delaying the TAI moment associated with GnRH treatment can be an alternative to improve the P/AI (Thomas, et al. J. Anim. Sci. v. 92, p.4189–4197, 2014). A total of 478 beef heifers [*B. taurus* (n = 368) and crosses (n = 110)] was used to compare TAI moment using conventional protocol of AI (48 or 60h) in females that did not display estrus until 48h after progesterone (P4) device removal. Females belonged to three farms of central and western regions of Rio Grande do Sul State and presented body condition score (BCS) of 3.2 ± 0.5 (range 1 to 5) at beginning of protocol (Day 0). On Day 0, heifers were classified according to the presence of corpus luteum (with CL; n=392 and without CL; n=85) and received the insertion of P4 device previously used for 16 days was done (PRIMER®, Tecnopec) plus 2mg of estradiol benzoate (BE, RIC-BE®, Tecnopec) IM. On Day 8, P4 device was removed and 0,482mg of sodic cloprostenol (ESTRON®, Tecnopec) IM and 1mg of estradiol cypionate (cypionate HC®, Animal HertapeCalierSaude) IM were administrated. At this moment, females were marked with a paint stick (Raidl-Maxi, RAIDEXGmbH, Dettingen / Erms, Germany) in the sacral region. Heifers with clean sacral region 48 hours after P4 device removal were considered as displayed estrus and were immediately inseminated (Estrus-TAI48h; n=311). Heifers without estrus received 10µg GnRH (buserelin; Prorelin®) IM and were randomly assigned to be inseminated at 48 hours (NEstrus-TAI48; n=87) or at 60 hours (NEstrus-TAI60; n=79) after P4 device removal. The pregnancy diagnosis was performed 30 days after TAI by ultrasound. Statistical analysis was performed using PROC GLIMMIX SAS (SAS 9.3). The overall P/AI was 47.8% (228/478). There was no difference (P = 0.31) on P/AI among groups [Estrus-TAI48h = 49.5% (154/311), NEstrus-TAI60h = 49.4% (39/79) e NEstrus-AI48h = 40.2% (35/87)]. Despite of the similarity among treatments, these results suggest that it is possible to improve the P/AI in heifers that did not display estrus 48 hours after P4 device removal, delaying the TAI moment associated with GnRH. It is noteworthy that further studies including a larger number of animals are certainly required.

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A077 FTAI, FTET and AI

Evaluation of the effect of administering different commercial eCG on follicle growth and conception, and validation of its need in TAI protocols for postpartum Nelore cows

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Keywords: beef cows, equine chorionic gonadotropin, postpartum anestrus.

This study evaluated the effect of two different commercial equine corionic gonadotropin (eCG) on ovarian follicular growth, dominant follicle diameter (ØFD) and pregnancy rate of Nelore cows (*Bos indicus*, n = 943) with 30-50d postpartum and synchronized to timed artificial insemination (TAI). The study was conducted at Vera Cruz IV Farm, Barra do Garças - MT. At the beginning of the treatment (D0), cows received an intravaginal device with 1g progesterone (Cronipres®, Biogenesis Bagó, Brazil) and 2 mg of estradiol benzoate i.m. (Bioestrogen®, Biogenesis Bagó). On D8, the devices was removed and were administrated i.m. 150µg of D-cloprostenol (Croniben®, Biogenesis Bagó) i.m and 1 mg of estradiol cypionate (Croni-Cip®, Biogenesis-Bagó). At that time, cows were homogeneously allocated (according to body weight, BCS and ØFD on D8) to one of three experimental groups: Control (without eCG), Novormon (MSD Animal Health, Brazil, 300IU i.m.) or Ecegon (Biogénesis-Bagó, 300IU i.m.). The TAI was performed 54h after device removal. Ultrasound examinations were performed on D0 to verify uterine regression, on D8 and D10 to evaluate the ØFD (mm; n = 529), and on D40 for pregnancy diagnosis (n = 943). The daily follicular growth rate (mm/d) was calculated by the difference between the ØFD on D8 and D10 divided by two. Data were analyzed using PROC GLIMMIX from SAS. Cows average BCS was 2.80 ± 0.02. The ØFD on D8 was similar (P = 0.92) between control (11.3 ± 0.2; n = 178), Novormon (11.1 ± 0.2; n = 174) and Ecegon (11.3 ± 0.2; n = 177), demonstrating the homogeneity between groups immediately before the treatment. However, cows treated with Novormon (13.4 ± 0.2^a; n = 174) or Ecegon (13.5 ± 0.2^a; n = 177) had higher ØFD in D10 than control cows (12.8 ± 0.2^b; n = 178; P = 0.001). Besides, the total (from D8 and D10) and daily follicular growth rates were higher in groups Novormon (2.4 ± 0.1^a and 1.2 ± 0.05^a; n = 174) and Ecegon (2.3 ± 0.1^a mm and 1.2 ± 0.05^a; n = 177) compared to control group (1.6 ± 0.1^b and 0.8 ± 0.04^b; n = 178; P = 0.001), respectively. Similarly, pregnancy rate 35d after TAI was greater (P = 0.03) in cows treated with Novormon (52.1%; 160/307) and Ecegon (52.8%; 181/343) in relation to control cows not treated with eCG (43.3%; 127/293). Therefore, the efficacy of eCG to increase ovarian follicular growth and the size of the dominant follicle in TAI protocols was demonstrated, and it has enabled the increase of pregnancy rate in postpartum Nelore cows. Also, both commercial eCG tested (Novormon® and Ecegon®) were equally efficient.

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A078 FTAI, FTET and AI

Conception rates and pregnancy losses in embryo recipients treated with lecirrelin 13 days after FTET - preliminary results

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Keywords: *Bos indicus*, GnRH, resynchronization.

The use of GnRH analogues for resynchronization of females previously submitted to FTAI has been previously reported (Vianna and Gois, 2014, Anim. Reprod. 11, 353). This study compared the pregnancy per embryo transfer (P/ET) and pregnancy loss of embryo recipients treated or not with Lecirelin. In study 1, retrospectively data of 4263 crossbreed (Angus x Nelore) *in vitro* produced embryo transfer in Nelore cows recipients (ECC: 3.0 to 2.5 range 1-5) were used. All recipients were previously synchronized with progesterone (P4) intravaginal device (CIDR®, Zoetis, Brazil) and 2 mg estradiol benzoate (Gonadiol®, Zoetis). Nine days later, they received 12.5 mg PGF2α (Lutalyse®, Zoetis), 300 IU eCG (Novormon®, Zoetis) and 1 mg estradiol cypionate (E.C.P®, Zoetis) IM. The timed embryo transfer (TET) was performed 18 d after the beginning of the hormonal treatment. Thirteen days later, they received (G-P4+GnRH; n=810) or no (G-P4; n=3453) 25 μg of lecirelin (Gestran Plus, Tecnopec, Brazil) IM. Transrectal ultrasonography for pregnancy diagnosis was done 29 and 60 days of the gestation. In the study 2 was retrospectively analysed similar to the previous, however the produced embryos were from Nelore bred (n=2152, G-P4+GnRH, n=341, G-P4, n=1811). Data were analyzed by Chi-square test ($p < 0.05$). In the study 1, the P/ET at 29 days ($P \leq 0.001$) were higher G-P4 [47.0%, (1622/3453) than G-P4+GnRH [33.6%, (285/810)]. However at 60 days, the P/ET ($P = 0.11$) were similar [G-P4: 40.6% (1405/3453) and G-P4+GnRH: 33.2 (269/810)]. Pregnancy losses ($P \leq 0.001$) were lower in the G-P4+GnRH [5.6% 16/285)] than G-P4 [13.3% (217/1622)]. In experiment 2, the P/ET at 29 and 60 days of G-P4 ($P = 0.001$) were higher [29 days: 39.2% (710/1811) and 60 days: 33.1% (601/1811)] than G-P4+GnRH [29 days: 30.0% (102/341) and 60 days 26.6% (91/341)]. The pregnancy losses ($P = 0.28$) were similar in groups [G-P4 (15.3% 109/710), G-P4 + GnRH: 10.7% (11/102)]. These preliminary results showed that the application of lecirelin unexpectedly decreased P/ET in the two retrospectively analysis. However, the pregnancy loss was reduced after the GnRH analogue administration. Further prospectively studies are necessary to confirm these results.

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A079 FTAI, FTET and AI

Pregnancy rates in nonsuclked or postpartum Nelore cows treated with lecirrelina 13 days after ftet

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Keywords: beef cattle, embryo recipients, GnRH.

The aim of this study was to compare the embryo transfer per synchronized females (ET/treated), conception (P/transferred) and pregnancy (P/treated) in nonsuckled and suckled Nelore cows and subjected to timed embryo transfer (TET) using resynchronization with unknown pregnancy status. Nelore cows (n=3652) with BCS from 3.0 to 3.5 (range 1 to 5) were used as embryo recipients and allocated into two experimental groups: 1062 suckled cow 40 to 90 days postpartum (G-S) and 2590 nonsuckled cows (G-NS). All recipients were previously treated with P4 intravaginal device (CIDR®, Zoetis, Brazil) and 2 mg estradiol benzoate (Gonadiol®, Zoetis). Nine days later, they received 12.5 mg PGF2α (Lutalyse®, Zoetis), 300 IU eCG (Novormon®, Zoetis) and 1 mg estradiol cypionate (E.C.P®, Zoetis) IM. The TET was performed 18 d after the beginning of the hormonal treatment. Thirteen days after TET, they received 25 µg of lecirelin (Gestran Plus, Tecnopec, Brazil) IM. Pregnancy diagnosis was done 39 days after the start of hormone treatment and non-pregnant recepients with CL received an embryo (second TET). Data were analyzed by Chi-square test ($p<0.05$). The P/transfered was 47.5% (1232/2590) and 58.1% (617/1062); ($P\leq0.001$) G-NS and G-S, respectively. After treatment with lecirelin, the ET/treated of non-pregnant cows ($P\leq0.001$), P/transferred ($P=0.02$) and P/treated ($P\leq0.001$) were higher for G-S [87.8% (391/445); 39.3% (154/391) and 34.6% (154/445)] than for G-NS [78.7% (1070/1358), 33.0% (353/1070) and 26.0% (353/1358)]. Cumulative pregnancy rate at 21 days, considering both TETs was 72.5% (771/1062) for G-S cows and 61.1% (1585/2590) for G-NS ($P\leq0.001$). In conclusion, the resynchronization with lecirelin provided adequate ET/treated, P/transferred and P/treated rates in suckled and nonsuckled cows. Nelore suckled cows presented higher ET/treated, P/transferred and P/treated rates in relation to nonsuckled cows. Thus, using postpartum suckled cows as embryo recipients for TET may be an interesting animal category option.

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A080 FTAI, FTET and AI

Comparation between performance data of yearlings and 2 years old Nellore heifers - preliminary data

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Keywords: Nelore, precocious pregnancy, yearling pregnancy.

Aiming to diminish the backgrounding of the heifers associated to usage of genetic selection to precocious calving (PC), yearling Nelore heifers have been breeding to become pregnant at this early age. The PC has positive correlation with economic carachteristics that impact the beef operation system. The aim of this trial was to evaluate retrospectively the productive data of heifers that became pregnant between 12 to 16 montns (Precocious = P) or 24 to 27 months (Not precocious = NP). A total of 305 heifers born between September and November of 2010 and weaned between May and July of 2011 received a 600 grams/animal/day protein mixture between May and December. At December of 2011, all heifers received an induction of puberty protocol following timed artificial insemination (TAI) according to Rodrigues et al., (Theriogenology, 82, 760, 2014). After the TAI, heifers were exposed to bulls in a proportion of one bull for 30 heifers until March 2012 (2011/2012 breeding season; BS). Heifers that become pregnant in the first BS were classified as P (n=164) and heifers that were not pregnant were classified as NP (n=141). The NP heifers went to the next BS (2012/2013) with 24 to 27 months of age. All heifers were evaluated in the subsequents BS (2012/2013 and 2013/2014). The data was retrospectively compared two groups (P vs. NP) of heifers: number of gestations, calving rate and number of weaned calves, total weight (Kg) of weaned calf, pregnancy loss, calf mortality, weight of heifer at weaning, weight of heifer at beggining of BS and mature weight. Mature weight was achieved at appoximately 44 months of age. Data were analyzed using MIXED procedure from SAS. There was no difference between age of heifers of P and NP group (432 days = 14.1 mo). The P heifers presented higher number of gestations (2.3 ± 0.05 vs. 1.2 ± 0.06), number of calvings (2.1 ± 0.06 vs. 1.2 ± 0.07) and number of weaned calves (1.4 ± 0.04 vs. 0.7 ± 0.05) compared to NP group ($P < 0.05$), respectively. Total weight of calf weaned (Kg) was higher to P group (312.5 ± 10) compared to NP (146.8 ± 11). Pregnancy loss (%) were higher for P (14.0) group compared to NP (4.3). There was no difference in calf mortality (10.5%), weight of heifers at weaning (214.1 ± 1.8), at beggining of BS (280.0 ± 1.7) and the mature weight (445.1 ± 3.6) of P and NP heifers. The pregnancy rates (%) at TAI and at end of BS when primiparous was 41.6^{a} and 67.9^{x} for P and 27.9^{b} and 60.4^{y} for NP, respectively. Weight of calves at weaning (Kg) for first and second parurition of P group was 211^{a} and 233^{b}, allthough was 206^{a} for primiparous cows of NP group. In conclusion, yearling Nelore heifers increase the total weight of calf weaned, even with higher pregnancy loss, and also not alter their mature weight. Further prospective studies certainly should be necessary to validate these results.

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A081 FTAI, FTET and AI

Circulating concentration of AMH in Holstein and Jersey breeds and its relationship to fertility in lactating cows and heifers

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Keywords: AMH, dairy cow, fertility.

The objective of this study was to verify whether the circulating concentration of the anti-Mullerian hormone (AMH) was associated with fertility in Holstein and Jersey lactating cows and nulliparous heifers. Blood samples were taken from the caudal vein/artery from lactating cows (n = 141 Holsteins and n = 82 Jerseys) and heifers (n = 407 Holsteins and n = 122 Jerseys) with evacuated tubes containing EDTA. Samples were immediately refrigerated and plasma harvested, placed into microcentrifuge tubes within 2h and frozen until AMH analysis. AMH analysis was performed with the MOFA Global (Bovine Fertility Assay®) kit. Lactating cows and heifers underwent daily estrus detection performed in the mornings and followed by AI. Non-pregnant animals received an Ovsynch-like timed AI protocol. Retrospective analysis of the reproductive parameters included: 1) interval from calving to conception for lactating cows; 2) age at conception for nulliparous heifers. Statistical analysis was performed with the Glimmix and Corr procedures of the SAS software (Version 9.3 for Windows). The circulating AMH concentration in lactating cows was 312 ± 47 and 318 ± 61 pg/ml for Jersey and Holstein respectively ($P>0.10$). However, Jersey heifers had greater circulating AMH than Holstein heifers (311 ± 31 pg/ml; 225 ± 24 pg/ml respectively; $P<0.05$). Despite of breed, circulating AMH was not associated with calving to conception intervals in lactating cows ($r = -0.05$, $P>0.10$) or age at conception in nulliparous heifers ($r = -0.06$, $P>0.10$). In conclusion, circulating AMH doesn't correlate with reproductive performance of lactating cows or heifers from Holstein and Jersey breeds. Further studies are needed using a larger number of animals to confirm these findings.

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A082 FTAI, FTET and AI

Progesterone concentration on pregnancy in Holstein cows enrolled in a TAI protocol based on P4/E2

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Keywords: fertility, primiparous, progesterone.

The present study evaluated if the progesterone (P4) concentration during ovulatory follicle development in a timed artificial insemination (TAI) protocol based on P4/E2 affects pregnancy per AI in lactating Holstein cows. Our hypothesis was that cows presenting greater P4 concentration have also greater fertility. To alter P4 concentrataion, females (n=594) were randomly assigned to receive one (n=310) or two (n=284) intravaginals devices of P4 (CIDR®, Zoetis, SP, Brazil). The TAI protocol utilized was: d-11 intravaginal device of P4 (new or previously used by 9 days) + 2mg im EB (Gonadiol®, Zoetis) + 100mcg im GnRH (Cystorelin®, Merial, SP, Brazil); d-4, 25mg im Dinoprost (Lutalyse®, Zoetis); d-2, 25mg im Dinoprost + 1mg im ECP (ECP®, Zoetis) + CIDR removal; d0, TAI. Females in the group 2CIDR received an adicional CIDR at d-11, which was removed at d-4. At d-11 (n=117), d-4 (n=351), d0 (n=214) and d10 (n=72), blood samples were taken from cows for P4 concentration determination. Pregnancy per AI was determined by ultrasound at d32 (DG1) and d60 (DG2). The binomial data were analyzed using PROC GLIMMIX and continuous data using PROC MIXED of SAS. An effect was considered significant when $P<0.05$ and tendency when $P<0.1$. P4 did not differ among treatments at d-11 (1CIDR=4.2±0.4 ng/ml; 2CIDR=4.5±0.4 ng/ml; $P>0.1$), and at d-4 (1CIDR=3.5±0.2 ng/ml; 2CIDR=3.8±0.2 ng/ml; $P>0.1$). An interaction was detected between treatment and CL presence at the beginning of TAI protocol in P4 at d-4 (without CL and 1CIDR=2.7±0.3 ng/ml; 2CIDR= 3.6±0.3 ng/ml; $P<0.05$). There was no difference among treatments in pregnancy per AI and pregnancy loss between DG1 and DG2 (DG1: 1CIDR=26.1% [81/310] vs. 2CIDR=22.9% [65/284]; DG2: 1CIDR=23.9% [74/310] vs. 2CIDR=20.1% [57/283]; Pregnancy loss: 1CIDR=8.6% [7/81] vs. 2CIDR=10.9% [7/64]; $P>0.1$). An interaction was detected between [P4] at d-4 and parity (Primiparous=PP; Multiparous=MP) on DG1 ($P4 < 2.61$ ng/ml: PP=12.1% [16/69] vs. MP=10.1% [21/107]; $P4 \geq 2.61$ ng/ml: PP=37.7% [28/63] vs. MP=14.6% [30/113]; $P<0.05$), and on DG2 ($P4<2.61$ng/ml: PP=12.4% [14/69] vs. MP=8.4% [19/107]; $P4 \geq 2.61$ ng/ml: PP=34.2% [27/63] vs. MP=11.2% [27/113]; $P<0.05$). Exogenous supplementation of P4 in lactating Holstein cows submitted to a TAI protocol based on P4/E2 with an injection of GnRH at it beginning did not alter the pregnancy. The increase of P4 concentration during follicular development increases the fertility particullary in primiparous cows, but not in multiparous.

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A083 FTAI, FTET and AI

Progesterone concentrations and ovarian response after PGF2α administration at beginning of TAI protocol in *Bos taurus* beef heifers

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Fundación Irauy.

Keywords: monodose, progesterone concentrations, TAI.

This experiment was carried out to evaluate the effect of the administration of PGF2α at different moments of a progesterone (P4) and estradiol based treatment, on serum P4 concentrations, ovulatory follicle diameter and time of ovulation in *Bos taurus* beef heifers. Hereford heifers (n=25) with corpus luteum detected by ultrasonography and body condition score 4.5±0.1 (Mean±SEM, 1 to 8 scale) were used. Heifers were randomly allocated to one of two experimental groups: 1) two half PGF2α doses of 250 µg of cloprostenol (DL Ciclase, Syntex, Buenos Aires, Argentina) administered in the insertion and progesterone device removal or 2) a single dose of 500 µg of cloprostenol administered at the time of P4 device removal. Females received an intravaginal device during 7 days containing 0.5 g of P4 (DIB 0.5, Syntex) and 2 mg of estradiol benzoate (Gonadiol, Syntex). At the time of DIB removal, 0.5 mg estradiol cypionate (Cipiosyn, Syntex) and 400 IU of eCG (Novormon, Syntex), were intramuscularly administered. The P4 concentrations were determined daily from the beginning of the treatment until 24 hours after P4 device removal. Ovarian follicles were monitored every 12 hours by ultrasonography (WED-9618V, Well.D, Shenzhen, China) since DIB removal until ovulation time. Data were analyzed by GLM and presented as mean±SEM. According to the results, PGF2α administration at the beginning and at the end of treatment induced lower P4 concentrations during DIB treatment (3.9±0.4 vs. 7.1±1.1 ng/mL; $P<0.05$). Furthermore, a greater ovulatory follicular diameter at DIB removal (12.2±0.5 mm) compared with the group that received only PGF2α at the end of treatment (9.6±0.5 mm; $P<0.05$) was observed. At ovulation, follicular diameter showed a tendency to be larger in heifers treated with PGF2α at beginning and the end of treatment (14.4±0.4 mm) compared to heifers treated only at the end of treatment (13.1±0.5 mm; $P<0.1$). Ovulation rate were 100% (13/13) and 83.3% (10/12) for heifers with PGF2α at the beginning and the end vs. PGF2α those received only at the end of treatment ($P=0.43$). Heifers treated with PGF2α at the beginning (64.6±10.4 h) showed a shorter interval between the P4 device withdrawn and ovulation compared to those treated only on device removal (78.0±8.5 h; $P<0.05$). In conclusion, PGF2α administration at the beginning and progesterone device removal reduced the P4 concentrations during P4 device treatment, increased ovulatory follicular diameter at P4 device removal and reduces the interval between the withdrawal P4 device and ovulation in *Bos taurus* beef heifers.

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A084 FTAI, FTET and AI

Endogenous progesterone concentrations affect progesterone release from intravaginal devices used for estrus synchronization in cattle

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Keywords: artificial insemination, FTAI, progesterone.

The increasing use of timed artificial insemination (TAI) observed in Brazil in the last years was mainly due to the possibility of anticipating time to pregnancy, including in noncycling cows. In this context, a number of studies support the use progesterone (P4) devices in synchronization programs, but it represents 43% of the total cost of the protocols. In order to facilitate the application of the technique and reduce hormone costs, several authors propose re-utilization of this progesterone release devices (DLP), but the results are controversial and do not describe with precision the amount of remaining steroid in these devices after being used in cows at different luteal status. Thirty cyclic crossbred heifers were divided into three groups (n=10/group). In the G1 and G2 the DLP (DIB, MSD – Brazil) was inserted (D0) seven days after the induced ovulation with estradiol benzoate (Benzoato HC, Hertape – Brazil). Furthermore, 0.15 mg of D-cloprostenol (Veteglan, Hertape – Brazil) was administered three days after the DLP insertion to promote luteolysis in G2. In the G3, the luteolysis was induced with 2 doses of D-cloprostenol administrated 4 and 3 days before DLP insert aiming to maintain only the exogenous P4 source. The three groups remained with DLP for 8 days, simulating a conventional TAI protocol. After treatment, each group of three DLP's were separated and subjected to removal and weighing of grinding silicon. An extra group using a new device (G4) was added. The P4 extraction was performed with methanol and the samples were analyzed in duplicate by RIA (IM 1188, Immunotech Inc., Prague, Czech Republic). The G4 samples were considered as standard and used for extraction technique validation. P4 means were compared between groups using the Tukey test. The P4 concentration that remained into the P4 devices was 30.75 ± 1.13 ng/mL (a); 14.65 ± 1,65ng/mL (b); 10.96 ± 1.15 ng/mL (c); 7.91 ± 1.22 ng/ml (d) ($P<0.05$) for G4, G1, G2 and G3 respectively. In addition, the absolute value of the residue found were 1,000 mg (a); 478 mg (b); 349 mg (c) and 257 mg (d), for G4, G1, G2 and G3 ($P<0.05$), respectively. Thus, the amount of P4 releasing from DLP is influenced by endogenous concentration of this steroid. Animals with high levels of endogenous P4 determined greater residual of P4 into the DLP. Additionally, lower quantity of residual P4 is expected when noncycling females are treated.

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A085 FTAI, FTET and AI

Different doses of equine chorionic gonadotropin in TAI protocol on suckled *Bos taurus* beef cows: impact on follicle growth and occurrence of estrus

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Keywords: *Bos Taurus,* eCG, IATF.

This study evaluated the use of different doses (0, 300 or 400 IU) of equine chorionic gonadotrophin (eCG) administered at the moment at progesterone (P4) device removal and their impact on follicular growth and estrus occurrence on suckled *Bos taurus* beef cows. Cows from five commercial beef farms located in three distinguished regions of Rio Grande do Sul state, Brazil were used. At the onset of the synchronization protocol (D0), 966 cows with body condition score 2.77±0.02 (score 1-5) received an intravaginal P4 device (CIDR®, Zoetis, SP, Brazil) and 2 mg of estradiol benzoate IM (Gonadiol®, Zoetis). On Day 9, the P4 device was removed, and were administrated 12,5 mg of dinoprost tromethamine IM (Lutalyse®, Zoetis) plus 1mg of estradiol cypionate IM (E.C.P.®, Zoetis). At this moment, cows were randomly assigned to one of three treatments: Control (n = 323), 300 IU (n = 326) or 400 IU (n = 317). The TAI was 48 h later P4 device removal. The females had their tail-heads painted with chalk paint (Raidl-Maxi, Raidex GmbH, Dettingen / Erms, Germany) at the time of removed of P4 device. Females without mark at the time of TAI were considered as displayed estrous. Additionally, 435 cows in anoestrus (no corpus luteum at the time of device insertion P4; Control: n= 146; 300UI: n= 153; 400 IU: n= 136) had follicular diameter at the time of the remove device P4 and the TAI evaluated to determine follicular growth after treatment. Statistical analyses were performed using the GLIMMIX procedure of SAS and the data compared by orthogonal contrast (C): C1 (eCG effect) and C2 (effect of eCG dose). The eCG treatment increased the estrus occurrence (Control = 53.7%, 300 IU = 70.6% and 400 IU = 77.0%; C1; P<0.0004), however, there was no difference between the doses used (C2; P=0.58). Also, because of the similarity of follicular diameter at the time of P4 device removal (Control = 11.0 ± 0.2 mm, 300 IU = 10.7 ± 0.2 mm and 400 IU = 11.0 ± 0.2 mm; C1; P=0.32 and C2; P=0.38) and larger follicular diameter on the TAI of cows treated with eCG (Control = 13.5 ± 0.3 mm, 300 IU = 14.0 ± 0.2 mm and 400 IU = 15.1 ± 0.3 mm; C1 ; P<0.04 and C2; P=0.19) the follicular growth (mm/day) was greater (Control = 1.2 ± 0.1 mm, 300 IU = 1.6 ± 0.1 mm and 400 IU = 2.1 ± 0.1 mm; C1; P<0.0001) in cows treated with eCG, regardless of the administered doses (C2; P=0.33). According to the literature, most animals having estrus manifest follicle and have higher probability of pregnancy. Therefore, the treatment with eCG, regardless of the dose of 300 or 400 IU administered, increases the occurrence of estrus and follicular growth in suckled *Bos taurus* beef cows.

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A086 FTAI, FTET and AI

Different doses of equine chorionic gonadotropin on timed artificial insemination protocol in suckled *Bos taurus* beef cows: impact on fertility

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Keywords: bovine, eCG, FTAI.

This study evaluated the use of different doses (0, 300, or 400 IU) of equine chorionic gonadotropin (eCG; Novormon®, Zoetis, Cravinhos, SP, Brazil) at the time of withdrawal of progesterone device (P4; CIDR®, Zoetis), and its impact on pregnancy per artificial insemination (P/AI) in suckled Bos taurus beef cows. Cows maintained on a native pasture in five commercial farms located in three distinguished regions of Rio Grande do Sul / Brazil (Southeast, Southwest and Western center). At the beginning of protocol (D0), a total of 996 cows with body condition score of 2.77 ± 0.02 (scale of 1 to 5) received a P4 insert and 2.0 mg estradiol benzoate IM (EB; Gonadiol®, Zoetis). On D9, P4 insert was removed, 12.5 mg of dinoprost tromethamine (Lutalyse®; Zoetis) and 1 mg of estradiol cypionate (E.C.P.®; Zoetis) were administrated via IM. At this moment, cows were randomly assigned to receive one of three treatments: Control (n = 323; no eCG), 300 IU (n = 326) or 400 IU (n = 317) of eCG. The timed artificial insemination (TAI) was performed 48h after removal of P4. Additionally, a subset of cows (n = 718) were subjected to ultrasound examination at the time of insertion and removal of the P4 for evaluating the presence of the corpus luteum (CL). Pregnancy diagnosis was performed by ultrasound 30d after TAI. Statistical analysis was performed using the PROC GLIMMIX of SAS®, and the data were analyzed by orthogonal contrasts (C): C1 (eCG effect) and C2 (eCG dose effect). The eCG treatment increased the P/IA (control = 29.7%; 300 IU = 44.8% or 400 IU = 47.6%; C1; P <0.0001). However, no difference difference eCG doses (C2; P = 0.55) effect was found. In the subset of cows (n = 718) that had presence of CL evaluated, an interaction between cyclic status and treatment on P/AI (P = 0.05) was observed. Overall cyclicality rate was 21.2% (152/718). There was no effect of eCG in the cyclic cows [Control = 55.4% (31/56); 300 IU = 54.2% (26/48) or 400 IU = 52.1% (25/48)]. However, non-cyclic cows showed positive effect of eCG treatment [Control = 25.8% (47/182); 300 IU = 41.6% (82/197) or 400 IU = 49.7% (93/187)]. The eCG treatment (300 or 400 IU) administered at the time of P4 removal increased P/AI in anestrus suckled *Bos taurus* beef cows.

Acknowledgments: Zoetis, CAPES, Agropecuária Posto Branco – Fazenda Nova Esperança, Cabanha Aguada, Agropecuária Odair Gonzáles, Fazenda Três Angicos e Estância Nova Aurora.

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A087 FTAI, FTET and AI

Follicle diameter on which eCG induces effect over follicular growth and fertility of *Bos indicus* cows submitted to TAI

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Keywords: eCG, fertility, zebu.

The objective of this study was to identify the diameter of the follicle, at the moment of eCG treatment, on which the eCG treatement increase on the final follicular growth and pregnancy rate in Bos indicus cows submmited to timed artificial insemination (TAI). The study enrolled 556 lactating cows, with post-partum between 30 and 60 days and body condition score (BCS) of 2.89±0.02 (1 to 5 scale). In a unknow stage of the estrus cycle (D0) all animals received 2 mg of estradiol benzoate (Sincrodiol®, Ourofino, Brazil) and an intravaginal progesterone device (Sincrogest®, Ourofino). On D8 all cows received 500µg of cloprostenol (Sincrocio®, Ourofino), 1 mg of estradiol cypionate (E.C.P.®, Pfizer, Brazil) and the progesterone devices were removed. At this point, cows were assigned, according to the follicular diameter, in one of two treatments (Control or eCG). Animals from eCG (n=277) received 300IU of eCG (Folligon®, MSD, Brazil) and cows from Control (n=279) received no additional treatment. Cows received a TAI 48 hours after progesterone device removal. On the study, ultrasound exams were performed on D8 and D10 to measure the follicular diameter and on D40 for pregnancy diagnosis. All data were analyzed by GLIMMIX procedure of SAS. After evaluation of different follicular diameters at progesterone device removal, it was observed that eCG showed effect on follicular growth and pregnancy rate of cows with follicles smaller than 10.6mm and 9.4mm, respectively. Thus, in cows with follicles <10.6 mm, the eCG Group (1.35±0.09 mm/day) showed greater follicular growth when compared to Control Group (0.79±0.07 mm/day; P=0.001) and in cows with follicles <9.4 mm, eCG Group [37.4% (34/91)] presented higher pregnancy rate than the Control Group [15.7% (17/108); P=0,001]. In conclusion, treatment with eCG in TAI protocols increases final follicular growth and pregnancy rate in cows with follicles <10.6 mm and <9.4 mm at progesterone device removal, respectively.

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A088 FTAI, FTET and AI

eCG increases fertility of crossbred dairy cows submitted to FTAI early in postpartum

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Keywords: dairy cow, eCG, FTAI.

The aim of this study was to evaluate the effect of eCG on fertility of crossbred (*Bos taurus* x *Bos indicus*) lactating dairy cows submitted to a fixed time AI (FTAI) protocol between October 2013 and July 2014. A total of 679 cows, 492 multiparous and 187 primiparous, with an average milk production of 21.4 ± 7.6 kg/d and average of 124.6 ± 97.0 DIM was used. At a random day of the estrous cycle all cows received an intravaginal progesterone device (CIDR®, Zoetis, Brazil), 2 mg estradiol benzoate im (Gonadiol®, MSD, Brazil) and 100 µg gonadorelin (Fertagyl®, MSD, Brazil) in the morning (D0). At D7 morning, all cows received 150 µg D-Cloprostenol im (Sincrocio®, Ouro Fino, Brazil) and were randomly assigned into two groups: eCG (n = 340) – received 400 IU eCG im (Novormon®, MSD); and Control (n = 339) – did not receive eCG. At the afternoon of D8, devices were removed and all cows received 150 µg D-Cloprostenol im and 1 mg estradiol benzoate im. The FTAI was performed at D10 morning. Diagnosis and confirmation of pregnancy were performed 30 and 60 days after AI. Data were analyzed by logistic regression using the PROC GLIMMIX of SAS and the results are presented following the group order eCG and Control. On pregnancy per AI (P/AI), the eCG improved fertility at 30 (37.8% vs. 30.2%; P = 0.06) and 60 (31.9% vs. 25.1%; P = 0.08) days. However, pregnancy loss between 30 and 60 days (9.4% vs. 14.4%; P = 0.3) and 60 days to calving (9.2% vs. 12.2%; P = 0.5) did not differ between groups. In addition, no difference was observed in the twin calving between groups (4.1% vs. 2.4%; P = 0.5). Furthermore, there was interaction (P = 0.09) between the use of eCG and DIM. Cows treated with eCG and inseminated up to 70 days postpartum had higher P/AI at 30 (39.0% vs. 25.2%; P = 0.02) and 60 (32.8% vs. 21.3%; P = 0.05) days after FTAI. However, in cows with more than 70 DIM this did not happen at 30 (36.6% vs. 35.7%; P = 0.8) nor at 60 (31.1% vs. 29.9%; P = 0.7) days. It was concluded that the use of eCG on D7 of the protocol increased fertility of crossbred lactating dairy cows submitted to FTAI due to improvement in the P/AI of cows inseminated until 70 DIM. Additionally, the use of eCG on D7 of the protocol did not increase twinning rate.

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A089 FTAI, FTET and AI

Effect of progesterone concentration and the time GnRH administration on pregnancy rates in Holstein heifers treated with the J-synch protocol

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Keywords: GnRH, Holstein, progesterone.

The aim of this study was to evaluate the effect of different concentrations of progesterone (P4) in the intravaginal devices (DIV) and different times of GnRH administration to induce ovulation in dairy heifers treated with the J-Synch protocol. In Experiment 1, Holstein heifers (n = 80) were used. On Day 0, all heifers received 2 mg of estradiol benzoate (BE, Proagro Argentina), while half were treated with DIV with 0.5 g of P4 (Synkro, Proagro, Argentina) and the other half with a DIV with 1 g of P4 (DIB, Syntex SA, Argentina). On Day 6, DIVs were removed and all heifers received 150 mg D + cloprostenol (PGF; Sincronil, Proagro). Heifers were again subdivided to receive 10.5 mg buserelin acetate (GnRH, Pro Buserelin, Proagro) 56 or 72 h after DIV removal and all were inseminated at fixed time (FTAI) at 72 h. Follicular dynamics, ovulation and pregnancy rates at 32 days were evaluated by ultrasonography (Honda 101 V, 5.0 MHz). When ovulation occurred earlier when the GnRH was administered at 56 h (91.9 ± 3.2 h) than when it was given at 72 h (98.8 ± 3.2 h; P = 0.035). However, there was no difference in the time of ovulation between heifers treated with DIV 0.5 g (92.8 ± 2.2 h) or DIV 1 g P4 (97.9 ± 2.8 h; P = 0.126). There was an interaction between DIV and GnRH time (P = 0.018) in the diameter of the preovulatory follicle, being smaller for the subgroup DIV 1 g and GnRH 56 h (11.8 ± 0.4 mm) from the subgroup DIV 1 g and GnRH 72 h (13.4 ± 0.4 mm; p <0.05), while the other subgroups, DIV 0.5 g and GnRH 56 h (13.1 ± 0.4 mm) and DIV 0.5 g and GnRH 72 h (12.6 ± 0.4 mm) were intermediate. Finally, pregnancy rate was higher in animals treated with DIV 0.5 g (19/40; 72.5%) compared to animals receiving DIV 1 g (16/40; 40%; P = 0.006). Although there was no difference between heifers treated with GnRH at 56 h (55.0%) or 72 h (57.8%; P = 0.9). In Experiment 2, the pregnancy rate was determined in Holstein heifers (n = 417) that received DIV 0.5 g P4 and 2 mg EB on Day 0 and were divided into three groups. Heifers in the first two groups were treated with the J-Synch protocol, receiving GnRH at 56 or 72 h and received a FTAI at 72 h of DIV removal. Heifers in the control group were treated with a DIV for 7 days, PGF with the removal of DIV, 1 mg of EB on Day 8 and were FTAI at 56 h after DIV removal. There were no significant differences in pregnancy rates among heifers treated with the J-Synch protocol with GnRH at 56 h (82/137; 59.9%), those treated with the J-Synch protocol and GnRH at 72 h (89/140; 63.6%) and the control group (86/139; 61.9%; P = 0.816). In conclusion, although although DIV with low P4 content improve pregnancy rates in heifers treated with the J-Synch protocol the time of GnRH administration did not affect pregnancy rates. Finally, pregnancy rates to the J-Synch protocol was not different to that in the conventional 7-day protocol.

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A090 FTAI, FTET and AI

Effect of angiotensin-(1-7) on ovulation rate of goats submitted to fixed-time artificial insemination

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Keywords: ACE, artificial insemination, progesterone.

A previous study showed an increase in estradiol production near ovulation in sheep subjected to estrus synchronization protocol when applying enalapril maleate (inhibitor of the Angiotensin Converting Enzyme-ACE) in the last three days of estrus synchronization protocol (Costa et al., Reproduction in Domestic Animal, v.49, p. e53-e55, 2014). The mechanism of this response may be due to the increase of angiotensin-(1-7), a new type peptide of the renin-angiotensin system, which occurs as a consequence of ACE inhibition (Brosnihan et al., Brazilian Journal of Medical and Biological Research, v.37, p.1255-1262, 2004). In this study was evaluated the effects of angiotensin-(1-7) in ovulation rate of goats subjected to a timed artificial insemination (TAI) protocol when applied near the ovulatory period. All animals were subjected to protocol synchronization of oestrus and ovulation, with 60mg of medroxyprogesterone acetate (Progespon-Syntex, Luis Guill on, Buenos Aires, Argentina) in intravaginal sponges for 11 days. On the 9th day were applied im, eCG 300UI (Novormon-Syntex) and 125µg cloprostenol (Sincrocio-Ouro Fino, Cravinhos, São Paulo, Brazil). On 11 day the sponges were removed and 12 and 13 days (24 and 48 hours after sponge removal, respectively) the animals received the treatments according to the experimental groups: control group received 30µg/kg of cyclodextrin in 2ml distilled water per animal subcutaneously and angiotensin group also received subcutaneously 50µg / kg of association Ang-(1- 7)+cyclodextrin, corresponding to 20µg/kg of angiotensin (1-7), diluted in 2ml of distilled water. Cyclodextrins are complex carbohydrates used in pharmaceuts preparations just to give solubility to the product. The AI was performed 38 and 50 hours after sponge removal with frozen semen, come from breeders approved in andrological examinations. Seven days after the last AI were collected blood from the jugular vein of 39 animals (20 angiotensin group and 19 in the control group) using vacuum tubes without anticoagulant and gel tab. The serum was frozen at -20°C until analysis. Progesterone concentrations were determined by ELISA test (Interkit-BioCheeck, Foster City, CA, EUA). The results were analyzed using Fisher's exact test ($p < 0.05$). The control group showed 73.68% (14/19) of ovulation and the group angiotensin- (1-7) showed 80% (16/20). There was no statistical difference between the two treatments ($P = 0.72$). It is concluded that the application of angiotensin- (1-7) during the pre-ovulatory period, with only two applications, was not able to increase the ovulation rate of goats subjected to TAI.

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A091 FTAI, FTET and AI

Effect of associating estradiol benzoate and GnRH at the beginning of the timed AI protocol on fertility of high producing Holstein cows

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Keywords: dairy cow, estradiol benzoate , timed AI.

This study was designed to test the hypothesis that associating estradiol benzoate (EB) with GnRH at the beggining of the timed AI (TAI) protocol would improve conception results (P/AI) in high producing dairy cows. Holstein cows (n = 254) with average daily production of 41.3 ± 5.8 kg, body condition score of 2.9 ± 0.1 and at 158.3 ± 9.4 DIM were synchronized at their first postpartum AI and if found open at pregnancy diagnosis. Cows were randomized according to parity and corpus luteum (CL) presence at D0 one of the following synchronizatrion programs for TAI: 1) EBGnRH: D0 = 2mg of BE (Sigma-Aldrich® + insertion of P4 device (CIDR, Zoetis), D2 = GnRH (100 μg gonadorelin, Factrel, Zoetis), D7 = PGF2α (25mg dinoprost, Zoetis) and P4 device removal, D8 = second treatment with PGF2α, D10 = TAI performed simultaneously with a GnRH treatment, aproximately 72h after P4 device removal; 2) NOEBGnRH: similar protocol, without the EB treatment on D0. Pregnancy diagnosis was performed by ultrasound 30 days after AI. Statistical analyses were performed with the procedure Glimmix of SAS, 9.3. Cows receiving EB associated with GnRH at the begining of the TAI protocol had greater P/AI compared to cows that received only GnRH (42.0% vs 31.0%; $P<0.05$). In addition, there was no interaction between the type of protocol and parity (Primiparous: EBGnRH = 36.8% vs NOEBGnRH = 22.2%, P=0.11; Multiparous: EBGnRH = 43.5% vs NOEBGnRH = 41.2%, P=0.80) or CL presence (with CL: EBGnRH = 48.0% vs NOEBGnRH = 31.6%, P=0.06; without CL: EBGnRH = 32.7% vs NOEBGnRH = 30.0%, P=0.79). These results show a positive effect of associating EB and GnRH at the beginning of the TAI protocol; however, future experiments will be required to elucidate underlying physiological mechanisms linked to this improvement in fertility, possibly involved with an improved control of the follicle wave emergence.

Acknowledgments: Zoetis.

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A092 FTAI, FTET and AI

Effect of animal category on FTET efficiency in buffaloes

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Keywords: buffaloes, embryo, FTET.

The present study aimed to evaluate the effect of animal category (nulliparous, primiparous or multiparous) on fixed-time embryo transfer (FTET) efficiency in buffaloes. A total of 125 buffalo females was distributed according to the category into one of three groups: nulliparous (NG, n=42), primiparous (PG, n=27) and multiparous (MG, n=56). At a unknow stage of the estrous cycle (D0), all animals were submitted to the same FTET protocol, which consisted on the insertion of an intravaginal progesterone device (P4, 1g, Sincrogest®, Ourofino Agronegócio, Brazil) and the simultaneously administration of 2mg im (intramuscular) of Estradiol Benzoate (EB, Sincrodiol®,Ourofino Agronegócio). Nine days after (D9), the P4 device was removed and the animals received 530µg im of Cloprostenol sodic (Sincrocio®, Ourofino Agronegócio) plus 400 IU im of eCG (Folligon, MSD Saúde Animal, Brazil). On D11, all buffaloes received 20µg im of GnRH (Sincroforte®, Ourofino Agronegócio). The females were evaluated for the presence of CL eight days after the P4 devices removal (D17) and only the ones with CL were submitted to FTET. Ultrasonographic evaluations were performed (Mindray DP2200Vet, China) on the following days: D11, for measuring the diameter of the largest ovarian follicle; D17, to evaluate the diameter of the largest ovarian follicle, CL and ovulation rate and D40, for the pregnancy diagnosis. The binomial and continuous variables were analyzed by PROC GLIMMIX and MIXED of SAS, respectively. Significance was considered when $P \leq 0.05$. No differences were verified between categories (NG vs. PG vs. MG) regarding the diameter of the largest follicle on D11 (10.5±0.3 vs.11.1±0.5 vs.11.5±0.4 mm; P=0.25) and on D17 (10.5±0.4 vs.12.0±1.1 vs.11.7±0.6 mm; P=0.30), the CL diameter (16.1±0.3 vs. 15.4±0.9 vs.16.6±0.4 mm; P=0.24), the pregnancy per embryo transfered (38.5 vs. 40.0 vs. 27.5 %; P=0.56) and the pregnancy per treated (23.8 vs.14.8 vs.19.6 %; P=0.65). However, the ovulation rate differed between primiparous and multiparous (61.9^{ab} vs. 42.3^{b} vs. 71.4^{a} %; P=0.001). The results obtained in the present study allow concluding that is possible to use the FTET in the different categories (nulliparous, primiparous and multiparous) of buffalo females.

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A093 FTAI, FTET and AI

Effect of the presence of corpus luteum in lactating buffaloes on the response to the Ovsynch protocol during the breeding season (preliminary results)

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Keywords: buffaloes, cyclicity, Ovsynch.

The aim of the present study was to evaluate the effect of the presence corpus luteum (CL) of lactating buffaloes in response to the Ovsynch protocol during the breeding season (autumn and winter). A total of 99 buffaloes, averaging 5.3±0.2 births, BCC of 3.5±0.1 and 85.5±5.2 days postpartum, was divided into two experimental groups: group without corpus luteum (CL, CG; n=50) and group with CL (CLG; n=49). At random stage of the estrous cycle (D0), all buffaloes received an intramuscular (im) injection of 10µg of GnRH (Buserelin acetate, Prorelinn™, Innovare Innovare Biotecnologia e Saúde Animal, Monte Aprazivel, Brazil). On D7, the animals received the administration im of 0.53mg of PGF2α (Cloprostenol sodic, Cioprostinn™, Innovare Innovare Biotecnologia e Saúde Animal, Monte Aprazivel, Brazil). Two days after (D9), 10µg of GnRH (Prorelinn™) was administered im and 16 hours later, all buffaloes were submitted to TAI (D10). Ultrasound evaluations (Mindray DP-2200Vet; Shenzhen, China) were performed to determine the cyclicity of the animals (presence of CL on D0), ovulation rate to the first GnRH (Ov-1stGnRH; presence of CL on D9), the diameter of the dominant follicle (øDF) on D9 and D10, ovulation rate to the second GnRH (Ov-2ndGnRH, rating on D10 and D13) and conception rate (CR) on D40. The CL vascularization (central vascularization - CV, and peripheral vascularization – PV; 0 to 4 scale, where 0 represents the absence of vascularization and 4 the maximum vascularization) and the CL diameter (øCL) were evaluated by color Doppler ultrasonography (Mindray M5Vet; Shenzhen, China) on Days 17, 21 and 25. The comparison between variables was performed by the analysis of variance (ANOVA), using the GLIMMIX procedures of SAS™. The CV, PV and øCL variables were analyzed as repeated measurements, using the MIXED procedure of SAS™ through the effects of treatment, time and its interaction (Treat.*Time). Difference was considered when P<0.05. For all analyzed variables, the CLG showed superior results to CG: Ov-1stGnRH (89.8 vs. 42.0 %; P<0.01); øDF on D9 (13.4±0.3 vs.12.1±0.3 mm; P<0.01); øDF on D10 (13.9±0.3 vs.12.3±0.3 mm; P<0.01); Ov-2ndGnRH (87.8 vs. 52.0 %; P<0.01) and CR (65.3 vs. 20.0 %; P<0.01). Similarly, for the CL vascularization data, greater results were observed for CLG on Days 17, 21 and 25, compared to CG: CV = 2.8±0.2, 3.1±0.1, 3.3±0.1 vs.1.8±0.3, 2.0±0.3, 2.2±0.3 (Treat.: P<0.01, Time: P=0.02, Treat.*Time: P=0.93); VP = 3.2±0.2, 3.5±0.1, 3.6±0.1 vs. 2.3±0.3, 2.4±0.3, 2.4±0.3 (Treat.: P<0.01, Time: P=0.14, Treat.*Time: P=0.83); øCL = 17.3±0.5, 18.7±0.4, 19.2±0.5 vs. 15.2±0.8, 14.4±0.9, 14.1±0.9 mm (Treat.: P<0.01, Time: P=0.02, Treat.*Time: P<0.01). In conclusion, buffaloes cows presenting a CL at the beginning of the Ovsynch protocol have better ovarian responses compared to females without CL when performed during the breeding season.

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A094 FTAI, FTET and AI

The effect of prostaglandinF2α applied at the beginning of the estrus synchronization protocol with progesterone and estradiol in *Bos taurus taurus* heifers

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Keywords: follicle diameter, progesterone device, TAI.

This research evaluated the effect of prostaglandin given at the start of timed artificial insemination (TAI) protocol on the follicle diameter at TAI and pregnancy rate. *Bos taurus taurus* beef heifers with 14 e 24 months old, body condition score with 3.6 ± 0.01 (1 to 5), were maintained during the entire study in native pasture on western central region of Rio Grande do Sul/Brasil. At the beginning of the protocol (Day 0) the animals received 2 mg estradiol benzoate IM (RIC-BE®, Tecnopec, São Paulo, Brazil). The heifers were distributed in three treatments according to the use of different types of intravaginal progesterone devices (P4): T1 = new device (1g with P4, PRIMER®, Tecnopec), T2 = used device (PRIMER®, Tecnopec) and T3 = monodose device (0.75g of P4; PROCICLAR®, Hertape Calier, Juatuba, Brazil). Additionally, heifers were randomly distributed in six different treatments regarding if they received or not a single dose of PGF at D0: T1 (n=39), T1PGF (n=51), T2 (n=259), T2PGF (n=262), T3 (n=81) e T3PGF (n=70). In Day 8, it was administered 1 mg estradiol cypionate IM (EC; Cipionato HC®, Hertape Calier) and 0.150 mg d-Cloprostenol (PGF; Prolise®, Tecnopec) in all animals. All heifers were inseminated 48h (Day 10) after P4 removal. At the TAI moment, the follicular diameter was measured (FD). Bulls were introduced in the herd ten days after TAI and remained for 60 days. The ultrasound examination for pregnancy diagnosis was performed on D40. Follicular diameters were 10.18 ± 0.29 (T1), 10.65 ± 0.30 (T1PGF), 14.03 ± 0.27 (T2), 13.0 ± 0.21 (T2PGF), 13.72 ± 0.19 (T3), 13.03 ± 0.25 (T3PGF) (P=0.1). The pregnancy rate was lower on T1 and T1PGF (33.3% and 37.3%; respectively) when compared with the others treatments (57.4% (T2), 53.6% (T2PGF), 49.4% (T3), 47.1% (T3PGF) (P=0.019), respectively. The use of PGF at Day 0 did not influence the P/IA (P = 0.88). However the pregnancy rate was lower in T1 (35.6%; 32/90) when compared to T2 (55.5%; 289/521) and T3 (48.3%; 73/151) (P=0.0018). The P/AI did not differ between the 14 and 24 month-old heifers (50.2% and 50.6%, respectively) (P = 0.357). Besides that, the pregnancy rate at the end of breeding season was 86.7%, and was not affected by treatments or heifers' age. The use of PGF at the beginning on the protocol did not alter P/AI in beef heifers, and P/AI was satisfactory when used a monodose or previously used P4 device.

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A095 FTAI, FTET and AI

Effect of eCG (Syncro-Part) on follicle growth and P/AI of *Bos indicus* cows submitted to TAI

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Keywords: *Bos indicus*, eCG, TAI.

The objective of this study was to evaluate the effect of eCG (Syncro-part, CEVA, France) UI on follicular growth, diameter of the dominant follicle (ØFD), ovulation rate, CL diameter and P/AI of primiparous Nelore cows (Bos indicus), between 30 and 80 days post-partum, submitted to TAI. At unknow day of the estrous cycle (D0), all cows (n=587) received a intravaginal device with 1g progesterone and 2 mg of estradiol benzoate i.m. On D8, the device was removed and 500µg of D-cloprostenol and 1 mg of estradiol cypionate were administered intramuscularly. Simultaneously, cows were randomly distributed to one of three experimental groups: Negative Control (no eCG - administration i.m. of Syncro-Part injection vehicle only), Positive Control (administration i.m. of 300IU of eCG; Novormon®, Zoetis, Brazil) and Syncro-Part group (administration i.m. of 300IU of eCG; Syncro-Part, CEVA, France). Timed AI was performed 48h after progesterone device removal. A subset of cows (n=89) underwent ultrasound evaluations on D8 and D10 to assess the ØFD and follicular growth. On D18 another ultrasound evaluation was performed to measure CL diameter as well as to calculate ovulation rate. All cows received a further ultrasound evaluation on D40 for pregnancy diagnosis. The daily follicle growth rate was calculated by the difference between the ØFD on D8 and D10 divided by two. Data were analyzed using PROC GLIMMIX from SAS. The ØFD on D8 was similar (P = 0.82) among Negative Control (10.4±0.5 mm), Positive Control (11.1±0.2 mm) and Syncro-Part group (9.8±0.4 mm), showing that groups were homogenous before the treatment. As expected, cows treated with Syncro-Part had greater ØFD on D10 and ovulation rate (13.1±0.6^{a} mm and 83.3%^{a}) than Negative Control cows (11.7±0.7^{b} mm and 56.7%^{b}; P=0.03). In addition, on D10, the Positive Control group had intermediate ØFD and ovulation results (12.8±0.6^{ab} mm and 75.9%^{ab}) that were similar to both other groups. Moreover, daily follicle growth rate was greater in cows in the Positive Control (1.5±0.2^{a} mm/day) and Syncro-Part (1.7±0.2^{a} mm/day) than Negative Control group (0.7±0.1^{b} mm/day; P=0.01). In contrast, there was no difference on CL diameter on D18 among experimental groups (Negative Control - 21.5±1.0 mm; Positive Control - 23.4±0.9 mm and Syncro-Part - 22.0±0.7 mm; P=0.32). The P/AI 30d after TAI was greater in Positive Control [54.7% (104/190)] and Syncro-Part [53.3% (106/199)] groups compared to cows in Negative Control group [37.9% (75/198)]. In conclusion, the Syncro-Part and Positive Control groups increased ovarian follicle growth, ovulation rate and P/AI of *Bos indicus* cows submitted to TAI in comparison to the Negative Control group.

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A096 FTAI, FTET and AI

Efficiency of two progesterone implants containing different quantities of active principles

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UNIFENAS.

Keywords: cloprostenol, corpus luteum, progesterone.

This research evaluated the effect of two different intravaginal progesterone devices (IVP), with the same matrix, containing 1.2 and 0.6g of P4, used in Fixed-Time Artificial Insemination (FTAI) protocol, on ovarian and progesterone (P4) concentration. A total of 24 cyclic crossbreed heifers weighting between 270 and 450 kg, was divided into one of two treatments according to their weight. Females from G1 (n=12) received IVP with 1.2g of P4, while those from G2 (n=12) received IVP with 0.6 of P4. All animals were presynchronized to eliminate the presence or formation of corpus luteum (CL), avoiding the endogenous source of P4. For this, 10 days before the beginning of the synchronization, females received a norgestomet ear implant plus 5.0mg of Estradiol Valerate and 0.5mg of cloprostenol. A second dose of PGF was applied 3 days before the initiation of the synchronization protocol. The animals were evaluated by ultrasonography exam (Mindray – 2200 – 7.5 Mhz linear transrectal transducer) in order to confirm the absence of CL at Day 0. Also, other examination were daily performed from D2 to D10 to evaluate the follicular wave emergence and ovarian follicular dynamics. On the first day of synchronization (Day 0), IVP were inserted and 2 mg of Estradiol Benzoate (EB) were applied. On the Day 8, the IVP were removed and 1 mg of EB was administrated 24 h later. The P4 concentrations were determined by RIA (DPC Medlab® in the Unesp-Botucatu Endocrinology Laboratory) from blood collected before and after the IVP insertion. The P4 concentration and the follicular diameter were compared using the Shapiro-Wilk test. Data were analyzed by ANOVA. The differences between treatments were accessed by the Student "T" test and on different days by the Turkey test. The P values lower than 5% were considered significant. There were no differences in P4 concentration ($P>0.05$) between treatments. Regardless of treatment, a significant effect of time was found ($P<0.0001$). There was no difference on the day of new follicular wave emergency (G1 = 3.3±1.8 and G2 = 4.0±1.2) and on diameter of follicle on Day 10 (G1 = 12.2±0.6 and G2 = 12.9±0.5 mm). There was a similar follicular growth from Day 4 to Day 10 between groups. In conclusion, both IVP containing 1.2 and 0.6g of P4 are efficient to maintain satisfactory plasmatic P4 concentration, resulting in a quickly rise after insertion and sharp decline after its removal. Also, different amount of P4 in the device did not alter the plasmatic P4 profile, resulting in satisfactory control of follicular growth and synchronization of ovulation in cyclic heifers.

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A097 FTAI, FTET and AI

Reproductive efficiency of 24 months old primiparous Nelore cows and submitted to resynchronization programs after TAI

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Keywords: primiparous, synchronization, TAI.

The present study evaluated the reproductive performance of Nelore (*Bos indicus*) primiparous cows having the first parturition at 24 months (mo) and submitted to two consecutive timed artificial insemination (TAI) programs with 32 days interval between inseminations. The trial was performed in one commercial beef farm, located in Camapua – MS, Brazil. Nelore females (n = 1,028) from three different categories [cows with the first parturition cows at 24 mo (Primi24; n = 115); cows with the first parturition at mo (Primi36; n = 475) and cows with second parturition at 48 mo (Second48; n = 438)] were submitted to the same reproductive program. During early postpartum (30 to 60 days) females were synchronized to receive a TAI. All females received, on the first day of the treatment (Day 0) an intravaginal progesterone device (P4; Cronipress Monodose M-24®, Biogenesis Bagó) and 2 mg of estradiol benzoate (EB; Bioestrogen®, Biogenesis Bagó) IM. On Day 8, the P4 devices were removed and it was administered 300 IU of eCG (Novormon®, Zoetis) IM, 1 mg of estradiol cypionate (EC; ECP®, Zoetis) IM and 0.75 mg of cloprostenol (PGF; Croniben®, Biogenesis Bagó) IM. All females were inseminated 48h after P4 device removal (1st TAI). On Day 32, all females were resynchronized at unknow pregnancy status, by the insertion of a P4 device and the administration of 1 mg of EB IM. On Day 40, the P4 device was removed and the pregnancy diagnosis was performed by transrectal ultrasonography (Chison 9300VET, Kylumax, Brazil). Non-pregnant females received 300 IU of eCG IM, 1 mg of EC IM and 0.75 mg of PGF IM and they received a TAI 48 h later (2nd TAI). The pregnancy diagnosis of the second TAI was performed on Day 72. Statistical analysis was performed using the GLIMMIX procedure of SAS (SAS® 9.3 Institute Inc., Cary, NC, USA, 2003). Pregnancy rate after 1st TAI was similar between the different animal categories (Primi24 = 53.0 vs. Primi36 = 54.3 vs. Second48 = 56.6%; P = 0.62). Furthermore, similar pregnancy rate was also observed after the 2nd TAI (Primi24 = 38.9 vs. Primi36 = 41.5 vs Second48 = 46.6%; P = 0.22). Additionally, no difference was observed regarding the cumulative pregnancy (1st+2nd TAI) between categories (Primi24 = 71.3 vs Primi36 = 78.1 vs Second48 = 78.3%; P = 0.70). Therefore, primiparous Nelore cows calving at 24 and 36 mo of age and second parturition cows aged 48 mo at calving had similar reproductive efficiency. The present data indicated the possibility of reducing the age at first parturition of heifes, without compromising the subsequent reproductive performance.

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A098 FTAI, FTET and AI

Efficacy of different drugs on ovulation induction of dairy cows submitted to timed artificial insemination

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Keywords: Girolando, ovulation induction, TAI.

Among the main estradiol esters used in cattle timed artificial insemination (TAI) programs, estradiol benzoate (EB) and estradiol cypionate (EC) has been commonly used because they low cost. But the estradiol estres present differences regarding their indication due to display different half-lives. In order to reduce management in TAI protocols, this study evaluated the follicular dynamics in dairy cows treated with EB and EC as drugs for ovulation induction. Therefore, 44 lactating Girolando dairy cows with average body condition score of 3 (range 1-5) were used. At random stage of the estrous cycle, the cows received an intravaginal device containing 1g of progesterone (DIB®, MSD Animal Health, São Paulo, Brazil) and 2mg of EB intramuscular (im) of which was considered day 0 (D0). At D8, the intravaginal device was removed and 150µg cloprostenol (CIOSIN®, MSD Animal Health) were applied (im) in all females. At this time the animals were randomly assigned into one of two treatments: EB Group (n = 22) and EC group (n = 22). The EC group received 1mg of EC (im) (ECP®, Pfizer, São Paulo, Brazil) at the time of P4 device removal in order to induce ovulation, while the EB group received 1mg of EB (im) (Estrogin®, Agroline, São Paulo, Brazil) 24 hours later (D9). After P4 device removal, ultrassonographic evaluations were performed every 12 hours until the ovulation. The following parameters were evaluated: diameter of ovulatory follicle - OF (mm); ovulation rate - OR (%) and P4 device removal/ovulation interval - ROI (h). Statistical analysis was performed using chi-square test for OR and "t" test for OF and ROI, with a 5% significance level. The results for EB and EC groups were, respectively: OF: 13.82±0.41mm and 13.86±0.61mm; OR: 86.36 % and 81.82%; ROI: 69.16±1.80 h and 64.82±2:58 h. There was no statistical difference ($p>0.05$) between treatments for any evaluated parameters. The results of this study corroborate with Sales et al. (Theriogenology, v.78, p.510-516, 2012) that observed no difference between the benzoate and cypionate for synchronization of ovulation in Nellore cows. This study also found greater dispersion of ovulations in the group treated with estradiol cypionate as noted in Martins et al. data (Acta Scientiae Veterinariae, v.33, p.285, 2005). Preliminary results of this study allow us to conclude that both drugs tested for ovulation induction can be used in TAI of Girolando dairy cows with the same efficiency.

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A099 FTAI, FTET and AI

Estradiol, GnRH or their association at the beginning of the resynchronization protocol results in similar reproductive efficiency in dairy cows

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Keywords: estradiol, lactating cow, resynchronization.

Two experiments were performed aiming to evaluate the reproductive efficiency of dairy cows undergoing resynchronization after TAI (Exp 1) and the effect of resynchronization on the corpus luteum (CL) blood flow (Exp 2). The Exp 1, enrolled 601 cows distributed into 3 groups, EB (n=200), GnRH (n=197) and EB+GnRH (n=204). Following previous TAI all females were resynchronized with the insertion of a P4 device (Primer®, Tecnopec) on Day 25 (EB and EB+GnRH groups) or on Day 26 (GnRH Group). Concomitantly with the P4 device insert, females of EB group received 2mg of EB (RIC-BE®, Tecnopec), GnRH group, 25µg of lecirelin (Gestran®, Tecnopec, Brazil) and group EB+GnRH, 2mg of EB and 25µg of lecirelin. On Day 33 all devices of P4 were removed and females of both experimental groups underwent an ultrasonography exam in order to assess pregnancy diagnosis from the 1ª TAI. All animals classified as non-pregnant received 0.282 mg of sodium cloprostenol (Estron®, Tecnopec) on Day 33, 1mg of EB on Day 34 and TAI on Day 35 AM. The pregnancy diagnosis reconfirmation of the 1st TAI and pregnancy diagnosis of the resynchronization was performed on Day 65. Statistical analysis was performed using the GLIMMIX procedure of SAS®. The experimental groups had similar pregnancy rate following 1st TAI, both at 33 [BE: 33.0, GnRH: 35.0 and BE+GnRH: 34.3%; P = 0.61] and 65 days [BE: 26.0, GnRH: 28.9 and BE + GnRH: 26.5%; P = 0.26]. Additionally, similar pregnancy loss was also observed between 33 and 65 days of pregnancy after 1st TAI [BE: 21.2, GnRH: 17.4 and BE+GnRH: 22.9%; P = 0.47]. Finally, the pregnancy rate 30 days after TAI related to the resynchronization was also similar between groups [BE: 20.9, GnRH: 17.2 and BE+GnRH: 25.4 %; P=0.54]. For all analyzed variables it was observed farm effect (P<0.05), however with no farm and treatment interaction (P>0.05). In Exp 2, 42 lactating cows used in the previous experiment [EB: n=15; GnRH: n=12; EB+GnRH: n=15] were evaluated by Doppler ultrasonography every 48h, between Days 25 and 33, to access CL vascularization according to the resynchronization protocol. Statistical analysis was performed using the GLIMMIX procedure of SAS®. No treatment effect (P=0.77), time (P=0.11) and interaction (P=0.81) between treatment and time was observed for CL vascularization rate. Therefore, the CL vascularization rate remained similar between the experimental groups during the experimental period (Day 25: 76.1, Day 27: 79.1, Day 29: 77.5, Day 31: 76.3 and Day 33: 77.1%). In conclusion, the resynchronization using 2mg of EB, 25µg of lecirelin or a combination of both after TAI results in similar pregnancy rate after 1st TAI and after resynchronization. Still, EB administration at the beginning of resynchronization protocol does not compromise the CL vascularization of females previously inseminated.

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A100 FTAI, FTET and AI

Factors affecting vaginal temperature in high producing lactating Holstein cows

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Keywords: milk production, pregnancy per AI, Heat stress.

The aim of this experiment was to evaluate the continuous collection of vaginal temperature for 72 h (every 10 min) in lactating Holstein cows (n= 480; 591 data collections of insemination) allocated in one single cross-ventilation barn. Cow were fitted with a thermometer attached to a progesterone intravaginal device (1.9g P4, CIDR, Zoetis) prior to timed artificial insemination (TAI). Milk production, DIM, skin thickness, coat color, body condition score (BCS), and pregnancy per AI (P/AI) data were collected and recorded for further analyses. Ambient temperature and humidity of barn was measured for 72 housing a data logger for calculation of the temperature and humidity index (THI). Data was analyzed using ANOVA and Pearson's correlations using proc GLM, Corr and Logistic of SAS. Maximum THI (MAX) and percentage of time above a vaginal temperature of 39.1°C (PCT) were used as data logger responses, class variables were created for MAX and PCT (High vs. Low) using the median threshold. There was a low correlation between THI and PCT ($P < 0.05$) ($r = 0.01$). Skin thickness was also poorly correlated with PCT ($P < 0.05$) ($r < 0.01$). Cows with black color coat spent less time with high vaginal temperatures ($P = 0.05$). Primiparous ($P = 0.04$) and cows with low BCS ($P < 0.01$) had greater PCT. Milk production was affected by parity ($P < 0.01$) and PCT ($P = 0.02$; High = 43.5 vs. Low = 41.2 kg/d). There was a milk production by MAX interaction for PCT ($P < 0.01$), whereas only cows in the highest milk production quartile (> 50.7kg/d) spent more time with high vaginal temperatures. Among the independent variables included in the model, parity ($P < 0.01$), PCT ($P = 0.03$; 26.1 vs. 17.4% for Low and High, respectively) and a PCT by milk production interaction ($P = 0.05$) affected P/AI. The decrease in P/AI in cows with High PCT only occurred in cows with the highest production. In summary, there is a large variability in individual capacity of cows respond to heat stress. Parity, BCS, coat color and milk production affected PCT, particularly under high ambient temperatures. Selection of animals with efficient control of body temperature in spite of high milk production should be further approached as a strategy to maintain adequate fertility of dairy herds.

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A101 FTAI, FTET and AI

Bacterial frequency of uterine samples from Mangalarga Marchador mares

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Keywords: endometritis, microbiologic, susceptible mares.

The endometritis in mares is considered one of the most frequent causes of infertility, ranked as the third most important disease in this species. Etiological agents of endometritis in the equine species can be Streptococcus equi subsp zooepidemicus, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans, and others (LANGONI et al., Revista Brasileira de Reprodução Animal, 23, 44-51, 1999). However, according to LeBlanc (Reproduction in Domestic Animals, 45, 21-27, 2010), the therapy is based, among other aspects, in the neutralization of virulent bacteria in controlling inflammation after breeding, which makes the identification of the etiologic agent essential for successful treatment. Therefore, this study aimed to identify the frequency of the possible etiologic agents of endometritis in the studied group of mares. Thus, 22 uterine samples obtained by sterile swab kept in Stuart transport medium of mares Mangalarga Marchador aged three and 20 years, with a history of subfertility and positive endometrial cytology for endometritis were used. Results were analyzed using descriptive analysis to calculate the absolute and relative frequencies (SAMPAIO, Estatística Aplicada à Experimentação Animal, 1998). Of the 22 samples analyzed, 14 (63.6%) were positive for microbiological examination, results similar to that found by Oliveira et al. (Veterinary Medicine, 1, 19-25, 2007) who obtained 64.4% (41/64) of positive mares in their study. The most common microorganisms were Enterococcus sp (18.2%), Escherichia coli and Staphylococcus sp (13.6%), Micrococcus sp (9.1%), Streptococcus sp and Serratia sp (4.5%). The frequency of Enterococcus sp and Escherichia coli identified in this study may be due to anatomical defects in perineal and vulvar region in consequence to advanced age of some mares, predisposing to pneumovagina and fecal contamination. According to LeBlanc (Proceedings of Reproductive Pathology, 78-84, 1997), it provides greater frequency of bacteria commonly found in the intestine. In conclusion, it was possible to identify the frequency of causing agents for endometritis in the studied group of mares.

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A102 FTAI, FTET and AI

Pregnancy associated glycoprotein predicts pregnancy losses in dairy cattle

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Keywords: dairy cows, glicoprotein, pregnancy loss.

The objective of this study was to determine the association between concentrations of pregnancy associated glycoprotein (PAGs) and pregnancy loss in lactating dairy cattle following fixed-timed artificial insemination (TAI) or timed embryo transfer (TET). Three experiments were performed, on experiment 01 (TAI - artificial insemination on d0) and 02 (TET - embryo transfer on d7) the pregnancy diagnosis was performed by ultrassonography at d31. In pregnant cows blood samples were collected and serum concentrations of PAG were determined by ELISA. On d59, a second pregnancy check was performed and the pregnancy loss was determined when a cow had a viable embryo on d31 of gestation but not on d59. In study 03 (TET - TE d7) cows were considered pregnant based on the presence of a vascularized corpus luteum on d24 (evaluated with a color doppler US) ipsilateral to the CL at TET, on days 31, 38, 45, 52 and 59 the cows were scanned to evaluate a viable embryo/fetus and blood samples were collected to evaluate the progesterone concentration (P4) and PAGs. One-way ANOVA (SAS 9.4) was used to evaluate differences of PAGs among d31, the LOGISTIC procedure was used to determine the probability of pregnancy maintenance. To test of the effectiveness of a single circulating PAG sample on d31 to predict pregnancy loss, a receiver-operating characteristic (ROC) curve was generated on MedCalc software package to determine PAG concentrations on d31 that should predict pregnancy loss with ≥95 accuracy in both TAI and TET. A total of 77 cows experienced pregnancy loss on TAI (exp 01/n=413) and 47 cows experienced pregnancy loss on TET (exp 02/n=238). Cows that were pregnant at d31 and maintained the pregnancy until d59 had higher ($P<0.05$) circulating concentrations of PAGs (AI=9.58±0.31; ET=8.61±1.53) compared to cows that experienced pregnancy loss (AI=4.15±0.33; ET=3.78±0.58) between d31 and 59 of gestation in both TAI and TET. Lower PAGs concentration on d31 resulted in higher ($P<0.05$) pregnancy loss until d59 of gestation (linear). Circulating concentration of PAGs below 1.4 ng/mL (TAI; minimal detectable level 0.28 ng/mL) and 1.85 ng/mL (ET) was 95 % accurate in predicting EM (between d31-d59) at d31 of gestation. On experiment 03, no effect was detected ($P>0.05$) of PAGs and P4 concentration on d24 in pregnancy maintenance until d31. The P4 concentration at d31 not interfere ($P>0.05$) in pregnancy maintenance; however, there was effect ($P<0.05$) of PAGs at d31 in pregnancy loss, as observed in experiments 01 and 02. The majority of pregnancy losses occurred between 24 and 31 (n=20; 20%), followed by days 31 and 38 (n=8, 10%), 38 and 45 (n=6, 7.5 %) and 45 to 59 and (n=1, 1%). In conclusion, PAGs concentration on d31 of pregnancy can be successfully used to detect pregnancy losses occurring after d31.

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A103 FTAI, FTET and AI

Timed artificial insemination according to diameter of the preovulatory follicle improves fertility in lactating beef cows

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Keywords: cattle, fertility, TAI.

The objective of this study was to evaluate whether timed artificial insemination (TAI) according to the diameter of the dominant preovulatory follicle (POF) would improve pregnancy rates in beef cows. In this study, lactating Nelore cows (Bos indicus; n=413), with 430-640 kg body weight, which were submitted to an estradiol/progesterone-based TAI protocol. On Day 0, cows given a progesterone device (1.9g Progesterone; CIDR®, São Paulo, Brazil) plus 2 mg of oestradiol benzoate (Bioestrogen®, Curitiba, Brasil) im, to synchronize follicular waves. On Day 10 (07:00 a.m.), the diameter of the POF was assessed by ultrasonography, and cows were randomized into Control (n=209) and Block (n=204) Groups. Control-Group cows were inseminated 48 h after CIDR removal, soon after ultrasound examination (08:00 a.m. on Day 10), and Block-group cows were inseminated at four time points according to the diameter of the POF as described by Pfeifer et al. (Reproduction, Fertility and Development, abstract, p 97, 2015): B0 (POF ≥ 15 mm, TAI at 08:00 a.m. on Day 10, n=51), B1 (POF 13 to 14.9 mm, TAI at 02:00 p.m. on Day 10, n=70), B2 (POF 10.1 to 12.9 mm, TAI at 08:00 a.m. on Day 11, n=54), and B3 (POF ≤10 mm, TAI at 02:00 p.m. on Day 11, n=28). Pregnancy status was assessed 30 days post-AI by ultrasonography. The diameter of the pre-ovulatory follicles on Day 10 was analyzed by analysis of variance – Factor ANOVA and the means between groups were compared by Tukey's post hoc test. Pregnancy rate was evaluated by Chi-square analysis. There was no difference in the diameter of the POF 48h after CIDR removal (P=0.4) between Groups. The pregnancy rate was greater in the Block-Group (63.2%, 129/204) than in the Control Group (48.8%, 102/209; P=0.002). In conclusion, our results demonstrate that TAI results can be improved when performed according to the diameter of the preovulatory follicle. This technique may represent an effective tool to improve fertility of lactating beef cows receiving TAI protocols utilizing estradiol as ovulation inducer.

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A104 FTAI, FTET and AI

Impact of equine chorionic gonadotropin associated with temporary weaning, estradiol benzoate, or estradiol cypionate on timed artificial insemination in primiparous *Bos indicus* cows

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Keywords: beef cattle, calf removal, eCG, TAI.

This study aimed to determine the impact of equine chorionic gonadotropin (eCG) associated with different timed-artificial insemination (TAI) protocols on the pregnancy rate (PR) in Bos taurus indicus previously treated with progesterone (P4). Five hundred and fifty-seven primiparous cows received the following treatments: on day 0 (d0), GeCGTW (group eCG+Temporary Weaning(TW); n=178) received 0.558g intravaginal progesterone (P4)+1.0 mg of estradiol benzoate (EB)(IM); on d8 (P4 removal+0.075 mg D-cloprostenol+400 IU eCG+TW for 48h); on d10, TAI+calf return to dam; GeCGEB (group eCG+EB; n=176) the same as GeCGTW without TW+1.0 mg of EB on d9; GeCGEC (group eCG+EC;n=203), the same as GeCGTW without TW+1.5 mg estrtadiol cypionate (EC) (IM). On d35 after TAI pregnancy diagnosis (PD) was performed. Open cows remained with clean-up bulls for 90 days and then underwent pregnancy diagnosis by ultrasound. Drugs used were from Biogenesis Bagó (Curitiba, PR) except eCG (Foligon, MSD, SP) and the EC (Zoetis, SP). The statistical analysis was performed with Chi-square (level of $P<0.05$). The PR of TAI was 51.1, 47.1 and 47.8% for GeCGTW, GeCGEB, and GeCGEC ($P>0.05$) respectively. The PR following clean-up bulls was 88.3, 47.3, and 31.1% ($P<0.05$). The final PR (TAI+clean-up bulls) of the groups was 94.4, 72.1, and 64.0%, respectively ($P<0.05$). In conclusion, there were no detectable differences in PR among TAI protocols; PR in the GeCGTW protocol following clean-up bulls was greater compared to others ($P<0.05$); the overall PR of cows subjected to TAI+clean-up bulls was significantly greater in GeCGTW as compared to other groups.

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A105 FTAI, FTET and AI

Impact of hormonal modulation of proestrus on uterine gene expression associated with cell proliferation of suckled anestrous beef cows

M.F. Sá Filho[1], A.M. Gonella-Diaza[1], G. Pugliesi[1], M. Sponchiado[1], M.F. Mendanha[1], R.S. Ramos[1], M.D. Goissis[1], F.S. Mesquita[2], P.S. Baruselli[1], M. Binelli[1]

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Keywords: equine chorionic gonadotrophin, estradiol cipionate, timed artificial insemination.

Previous studies demonstrated that estradiol cypionate (ECP) supplementation at the onset of proestrus alters uterine transcriptome of anestrous suckled beef cows (Sá Filho et al., IETS, 2015. p. 102 – abstract). Enriched pathways affected by the ECP supplementation described in this previous work identified genes associated with cell proliferation, suggesting a relationship with the modulation of uterine receptivity. Thus, the aim of the present study was to evaluate the impact of ECP and/or equine chorionic gonadotropin (eCG) supplementation at the onset of synchronized proestrus on endometrial expression of candidate genes associated with cell proliferation, in suckled anestrous beef cows. Evaluated genes were: progesterone receptor (PGR), oestrogen receptor 1 (ESR1), oestrogen receptor 2 (ESR2), epidermal growth factor receptor (EGFR), heparin-binding EGF-like growth factor (HB-EGF), collagen, type IV, alpha 1 (COL4A1), patched homolog 2 (PTCH2) and integrin, beta 3 (ITGB3). A total of 46 suckled cows was treated with 2 mg of intramuscular estradiol benzoate and received a P4 intravaginal device. Eight days later, P4 devices were removed, and cows received an intramuscular administration of 500 mg of cloprostenol. Cows were blocked by body condition score and diameter of largest follicle (LF) at the time of progesterone (P4) device removal. Simultaneously, animals were randomly assigned to one of the following groups: control (CON; n=11), ECP (1mg; n=11), eCG (400IU; n=12) and ECP+eCG (1mg and 400IU, respectively; n=11). At 48 h after the P4 device removal, all cows received 10 μg of buserelin acetate and were immediately artificially inseminated at fixed time. Six days after GnRH treatment, cows that presented a recently formed corpus luteum had uterine tissue collected by transcervical biopsy. The P values < 0.10 were considered as statically different. There were no interactions (P>0.10) between ECP and eCG on the expression of the evaluated transcripts. ECP treatment induced greater endometrial abundance of PTCH2 (P=0.07) and COL4A1 (P=0.02) genes, whereas suppressed EGFR (P=0.09) gene expression. ECP treatment did not affect the gene expression of ESR1 (P=0.90), ESR2 (P=0.61), HB-EGF (P=0.80) and ITGB3 (P=0.57). On the other hand, eCG treatment induced greater endometrial abundance of HB-EGF (P=0.06), ESR2 (P=0.09), and ITGB3 (P=0.05) genes, whereas reduced the gene expression of ESR1 (P=0.05). eCG supplementation did not alter the expression of EGFR (P=0.34), PTCH2 (P=0.31) and COL4A1 (P=0.19). Additionally, the expression of PGR was not altered by either ECP (P=0.51) or eCG (P=0.25) treatments. Therefore, the present results support the hypothesis that supplementation with ECP or eCG at onset of the proestrous in suckled anestrous beef cows acts on the endometrial tissue to alter the abundance of genes associated with cell proliferation during early diestrus.

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A106 FTAI, FTET and AI

Impact of bovine genetic group (*Bos indicus vs. Bos taurus*) and level of dry matter intake (high vs. low) on gene expression of liver enzymes related to progesterone metabolism

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Keywords: liver, metabolism, progesterone.

The objective of this study was to evaluate the liver expression of six genes associated with hepatic progesterone (P4) metabolism (CYP2C19, CYP3A4, SRD5A1, SRD5A3, AKR1C4 and AKR1D1) in heifers of *B. indicus* (Nelore; n=16) and *B. taurus* (Holstein; n=16) breeds that were kept in a high (HDM, gaining 900 g/day) or low (LDM, maintenance, NRC 2001) levels of dry matter intake. Cycling heifers from both genetic groups and level of dry matter intake were randomized in a 2x2 factorial design. The diet was administered for 33 days prior to liver biopsy. Pre-synchronized heifers, on day seven after ovulation, received two doses of PGF2α 6 hours apart. Twelve hours after the last treatment all heifers received a new P4 device (CIDR®, Zoetis, Brazil), 2 mg of estradiol benzoate i.m. (EB, Sincrodiol®, Ourofino Agronegócio) and an additional PGF2α treatment. Forty eight hours after device insertion, liver tissue was collected for later gene expression analysis, in addition to simultaneous blood sampling for assessment of circulating P4. Liver samples were immediately stored in 1 mL of RNA-stabilizing solution (RNAlater Ambioc Inc., Austin, TX), and maintained at 4°C for 24h, and then at -80°C for further mRNA quantification by qPCR. Circulating P4 (mean±SEM) and gene expression were analyzed with the MIXED and GLIMMIX procedures, respectively, from SAS. There was an interaction between genetic group and diet for circulating P4 (P=0.07). Zebu heifers receiving HDM had lower circulating P4 than LDM (2.3±0.2 vs 3.4±0.2 ng/mL, P=0.002). In contrast, diet did not influence circulating P4 in *B. taurus* heifers (2.0±0.2 vs 2.3±0.2 ng/mL, P=0.4). In addition, *B. taurus* and *B. indicus* heifers receiving HDM had similar P4 concentrations, respectively (2.0±0.2 vs. 2.3±0.2 ng/mL, P=0.40). However, Nelore heifers on LDM had greater circulating P4 compared to *B. taurus* heifers on the diet (3.4±0.2 vs. 2.3±0.2 ng/mL, P=0.006). There were no interactions between genetic group and diet (genetic group*diet=P>0.10) for any of the evaluated genes. Interestingly, *B. taurus* heifers had greater expression of SRD5A1 (P<0.0001), AKR1C4 (P<0.0001) and AKR1D1 (P=0.002) than zebu heifers. However, the expression of CYP3A4 was greater in zebu than in *B. taurus* heifers (P<0.0001). Independently from genetic group, expression of AKR1D1 in heifers fed HDM was greater compared to LDM diet (P=0.004) whereas expression of AKR1C4 was greater in heifers on LDM compared to HDM (P=0.01). The expression of CYP2C19 and SRD5A3 was not influenced by genetic group (CYP2C19: P=0.76, SRD5A3: P=0.18) or diet (CYP2C19: P=0.27, SRD5A3: P=0.94). These results indicate that gene expression of enzymes related to P4 metabolism can be partially regulated by dry matter intake level as well as genetic group.

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A107 FTAI, FTET and AI

Impact of injectable long-acting progesterone and/or hCG three days after TAI on the conception rate of lactating Holstein cows

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Keywords: Holstein, injectable progesterone, timed AI.

The aim of the present study was to evaluate the effects of administration of injectable long-acting progesterone (P4LA), associated or not with hCG treatment after timed-AI on conception rates of dairy cows. The study was conducted in two commercial farms (Farm A: 27.4L of milk/cow/day and free stall system and Farm B: 40.9L of milk/cow/day and corss ventilation system), both in São Paulo, Brazil. On a random day of the estrous cycle, 908 Holstein cows received a synchronization of ovulation treatment [D0: P4 (Sincrogest®, Ourofino Agronegócio) + EB (2 mg, i.m., Sincrodiol®, Ourofino Agronegócio); D8: device removal + PGF (0.53 mg, im, Sincrocio®, Ourofino Agronegócio) + ECP (1.0 mg, im, SincroCP®, Ourofino Agronegócio); D10 AM: TAI]. Three days after TAI, the animals were homogeneously distributed according to the daily milk yield, days in milk, parity and body condition score and divided into four experimental groups (2x2 factorial): 1. Control, cows receiving no additional treatment (n=250); 2. P4LA-900, treatment with 900 mg i.m. of P4LA (Sincrogest injetável®, Ourofino Agronegócio; n=245); 3. hCG, treatment with 2,000 IU i.m. of hCG (Chorulon®, MSD Schering, hCG) and 4. P4LA+hCG, treatment with both treatments (n=250). Data analysis was performed with the procedure GLIMMIX of SAS 9.3®. There was no effect of hCG or an hCG-by-P4LA interaction for none of the evaluated variables. Greater conception rate 30 days after TAI (CR30) was observed in cows treated with P4LA-900 (34.3 vs. 26.6%; P=0.002), and these results were independent from hCG treatment. Both the conception rate at 60 days (CR60) and the rate of pregnancy loss between 30 and 60 days after TAI (PL) were not affected by P4LA-900 or hCG, P>0.05. There was an interaction between P4LA-900 treatment and farm for CR60 (P=0.09), being observed a positive effect of P4LA on farm A (27.9 [362] vs 37.2% [363]), but not on farm B (18.7 [123] vs 15.2% [132], without and with P4LA, respectively). Interestingly, there was an interaction between P4LA treatment and season (warm vs. not warm) on CR30 (P=0.04) and CR60 (P=0.07) after TAI. Greater CR was verified in cows treated with P4LA during the warm season of the year (CR30: 16.3 vs 30.2% and CR60: 15.2 vs 26.9%). However, this increase was not observed during cooler seasons of the year (CR30: 32.9 vs 36.7% and CR60: 31.9 vs 33.9% without and with P4LA, respectively). In conclusion, treatment with 900 mg of P4LA three days after TAI in lactating Holstein cows increased conception rate at 30 days, mainly during the hot season of the year. There was no effect of hCG on conception rate.

Acknowledgments: Fazenda Colorado; Fazenda JIDA and OuroFino Agronegócio.

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A108 FTAI, FTET and AI

Impact on the conception rate and expression of estrus using different GnRH or esters of estradiol as inducer of ovulation in TAI protocols in crossbred dairy cows

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Keywords: estradiol, Girolando, TAI.

The objective of this study was to compare the expression of estrus and conception rate with the use of different estradiol esters [estradiol benzoate (BE) or estradiol cypionate (EC)] or gonadotropin-releasing hormone (GnRH) as ovulation inducers in artificial insemination protocols for fixed time AI (IATF) in Girolando cows (Bos taurus x Bos indicus). At random stages of the estrous cycle (Day 0), a total of 618 cows with an average BCS of 3.04±0.02; DPP of 147.6±4.39, and average production of 22.4±0.4 L/day, received an intravaginal progesterone release device (P4) and 2.0 mg EB (IM, Gonadiol®, MSD Animal Health , São Paulo Brazil). On the removal of the P4 device (Day 8), cows received 530 mg of sodium cloprostenol (IM, Ciosin®, MSD Animal Health, São Paulo, Brazil), 400 IU equine chorionic gonadotropin (IM, eCG; Novormon®, MSD Animal Health, São Paulo, Brazil) and marked with tail-chalk to verify later manifestation of estrus. Then, all cows were allocated to one of the following experimental groups: 1) EC [n=204; 0.5 mg CE, IM (ECP®, Zoetis, Sao Paulo, Brazil), on the 8th and artificial insemination (AI) after 48h]; 2) EB (n=205; 2.0 mg BE, IM on days 9 and 24 h after IA) or 3) GnRH [n=209; 10mg Burserelin (Prorelin®), IM at TAI on day 10]. Cows that had the tail-chalk removed were considered to have showed estrus. Thirty days after AI the cows were submitted to ultrasonography for pregnancy diagnosis (transrectal linear transducer 5.0 MHz, CHISON D600VET, USProducts, Eletromedicina, Brazil), the pregnancy was characterized by observing the embryo's heartbeat. Statistical analyzes were performed using SAS PROC GLIMMIX software (SAS® 9.3). Estrus expression was greater in BE group when compared to the EC and GnRH groups, respectively (79.5 vs 60.6 vs 46.9%, P=0.0001), and similar between the EC and GnRH groups. However, there was no statistical difference among groups in terms of conception rate [BE=32.7% (64/196); EC=26.6% (52/198); GnRH and=26.7% (55/206), P=0.83]. Therefore it is concluded that the expression of estrus was greater following BE than CE and GnRH. In conclusion, the different ovulation inducers produced similar conception rates after TAI, although estrus expression was enhanced when estradiol esters were used as ovulation inducers.

Acknowledgments: Marajoara Farm, São Luiz Farm, MSD Animal Health.

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A109 FTAI, FTET and AI

First use progesterone implants on reproductive performance of Nelore heifers in a TAI protocol

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Keywords: corpus luteum, follicle, Zebu heifers.

Some studies indicate that high concentrations of plasma progesterone (P4) decrease LH pulsatility and, consequently, the follicular growth. As a result, high circulating P4 can decrease the efficiency of protocols for fixed time artificial insemination (TAI) that rely on utilization of intravaginal devices loaded with P4. This study aims to verify if first use P4 implants, loaded with 1.2 g of P4, will interfere with follicular dynamics in beef heifers receiving a TAI protocol. In this experiment Nelore heifers (n=33) without corpus luteum (CL) at ultrasound exam, were randomly distributed to receive one of the following treatments. At P4 device insertion day (D0) all heifers received 2 mg of estradiol benzoate (BE) and an intravaginal device with P4 0.6 g (monodose group, n=11); or a previously used for 8 days P4 device of 1.2g (used group, n=11); and finally a device with P4 1.2 g without previous utilization (Max group, n=11). Eight days later (D8), 2mL of PGF2α was administered, simultaneously to 300 IU of eCG and ECP 0.5 ml to all animals; then the device was removed. Throughout D0 to D17, were performed ultrasound exams to evaluate ovaries and measure follicular diameter at 4, 8 and 10 days, and the CL on day 17. Blood samples were collected for P4 measurement on 0, 2, 4, 8 and 17 days. The follicular diameter, CL diameter, and P4 concentration variables were analyzed for homogeneity and normality tests, and later compared by ANOVA test. Variables that presented $P<0.05$ were compared by the DUNCAM test. There were no statistical differences among groups when we compared the follicular diameter on 4, 8 and 10 days, as well as the CL diameter on day 17 ($P>0.05$). The monodose device (0.6 g of P4) released greater ($P<0.05$) amounts of P4 than the Max device (P4 1.2 g) and also than the Max device (previously used) on days 2 and 4. In conclusion, these results suggest that implants with greater amounts of progesterone (1.2g) do not appear to change follicular growth profile in Nelore heifers, and can be indicated to be used without previous utilization in Nelore heifers.

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A110 FTAI, FTET and AI

Ciclicity induction and reproductive efficiency of 14 months old Nelore heifers submitted to fixed time artificial insemination

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Keywords: AI, precocity, synchronization.

Protocols to induce cyclicity in zebu heifers with 24 months age increases cyclicity and conception rates after timed artificial insemination (TAI), however there is little information addressing that for Nelore heifers with 14 months age. The objective of the present study is to evaluate the influence of the protocol to induce cyclicity on reproductive efficiency of Nelore heifers. A total of 626 heifers with 14.0±0.04 months age, 261.3±0.94kg of live weight and BCS=3.05±0.01 (1-5), from two commercial beef farms (Camapuã and Três Lagoas, MS, Brazil) and distributed into 5 different cohorts were enrolled in the trial. Females were randomly allocated, within each cohort, into two experimental groups: Induction (n=307) and Control (no additional treatment; n=319). The Induction group received (Day -22) a progesterone (P4; CIDR®, Zoetis) device previously used for 24 days, which remained for 10 days. Immediately after P4 removal (Day -12), it was administrated 0.6 mg IM of estradiol cypionate (EC; E.C.P®, Zoetis). Twelve days after (Day 0) all heifers were submitted to the same TAI protocol, receiving a auricular ear implant containing 3 mg of Norgestomet (CRESTAR®, MSD) and 2 mg IM estradiol benzoate (EB; Gonadiol®, MSD). At Day 8, the ear implants were removed and it was administered 0.6 mg IM de EC, 0.265 mg IM of sodium cloprostenol (Ciosin®, MSD) and 300 IU of equine chorionic gonadotrophin (eCG, Novormon®, MSD). Heifers were inseminated 48 h after (Day 10; 1st TAI). At Day 32 (22 days after 1st TAI), all females were resynchronized to receive the 2nd TAI with the same protocol described above, except by the EB dose at beginning of treatment (1 mg). At Day 40, the ear implants were removed and the pregnancy was diagnosed by transrectal ultrassonography examination (Chison 8200VET, Kylumax). Non pregnant heifers received the same protocol used for 1st TAI. The second pregnancy diagnosis was performed at Day 72 to evaluate P/AI after 2nd TAI. Moreover, ultrassonography examinations were performed on Days -22, -12 and 0 to evaluate the presence of a corpus luteum (CL). Statistical analysis was performed using the PROC GLIMMIX procedure of SAS (SAS® 9.3). The CL rate at Day -12 was similar between groups (Induction=1.3 vs. Control=1.3%; P=0.95) however it was greater at Day 0 in heifers from Induction group (75.3%) in comparison to Control group (7.8%; P<0.0001). Regarding the P/AI, no differences were observed among groups after 1st TAI (Induction=43.0 vs. Control=42.9%; P=0.99), after 2nd TAI (Induction=35.6 vs. Control=32.6%; P=0.67) and accumulated (1st TAI + 2nd TAI; Induction=63.5 vs. Control=61.8%; P=0.75). Therefore, it is concluded that the protocol to induce cyclicity increases the percentage of cyclic Nelore heifers at 14 months of age; however, does not change P/AI after 1st and 2nd TAI.

Acknowledgments: São Mateus Farm, Engano Farm, Bovifértil and CNPq.

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A111 FTAI, FTET and AI

Influence of follicle diameter at the time of TAI on the occurrence of estrus and conception rate in *Bos taurus* beef cows in anestrus

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Keywords: *Bos taurus,* follicular diameter, TAI.

Follicular diameter at the time of artificial insemination at fixed time (TAI) is associated with the response of treatments for ovulation synchronization. Thus, the aim of the present study was to evaluate the diameter of the largest follicle (LF) present at the IATF time on the occurrence of estrus rate and the pregnancy rate per AI (P/AI) in anestrus lactating *Bos taurus* beef cows. Non-cyclic cows (n=435) from five commercial farms located in three different regions of Rio Grande do Sul state and kept on native pasture-grazing conditions were enrolled in this experiment. At the beginning of the synchronization protocol (D0), cows had an average of body condition score of 2.77±0.02 (scale of 1 to 5) and received a progesterone device (P4; CIDR®, Zoetis) and 2 mg IM of estradiol benzoate (EB; Gonadiol®, Zoetis). At D9, the device was removed and was administered IM 12.5 mg of dinoprost tromethamine (Lutalyse®, Zoetis) and 1 mg estradiol cypionate (ECP®, Zoetis). At this time, the females had the tail head painted with a paint stick (Raidl-Maxi, Raidex GmbH, Dettingen / Erms, Germany) to identify females that displayed estrus between device removal and TAI. On D11, cows were artificially inseminated and had the LF identified and measured by transrectal ultrasonography. Only females having no corpus luteum at the time of insertion and removal of the P4 device were used. Statistical analysis was performed using the SAS program. The average diameter of the LF was 14.2±0.2 mm. Analysis of the ROC curve (receiver operating characteristic), demonstrated that the critical LF diameter for the gestation establishment was 15 mm and the area under the curve was 66.1 [confidence interval (95% CI) was 0.661 to 0.780, P<0.001]. Cows that had LF > 15 mm (n=257; 17.0±0.1) had higher occurrence of estrus (84.8% vs. 63.0%; P<0.0001) and higher P/IA (53.9% vs. 22, 6% P<0.0001) than those with LF ≤ 15 mm (n=178; 12.5±0.1). Also, a linear relationship was observed between the LF diameter and the probability of estrus occurrence [exp (1.5503+0.1802*LF/1+exp(-1.5503+0.1802*LF), P<0.0001)] and P/IA [exp (-3.4719+0.1985*LF/1+exp(-3.4719+0.1985*LF), P<0.0001)]. There was no quadratic relationship between the LF diameter and the occurrence of estrus (P=0.19) or P/AI (P=0.30). Thus, as the LF diameter at TAI increases, so it does the occurrence of estrus and P/AI, being 15 mm the threshold size for pregnancy establishment in suckled non-cyclic taurine beef cows enrolled in synchronization of ovulation protocols for TAI.

Acknowledgments: Zoetis, Cabanha Aguada, Agropecuária Posto Branco, Agropecuária Odair Gonzáles, Fazenda Dois Angicos and Estância Nova Aurora.

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A112 FTAI, FTET and AI

Influence of uterine enviroment (lactating dam or nulliparous) and dam's fertility on future reproductive performance of their offspring in Holstein cows

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Keywords:, fertility, Holstein, uterine environment.

The objective of this retrospective study was to evaluate whether reproductive efficiency of the dam and/or its uterine environment (lactating dam or nulliparous heifer) would affect future reproductive efficiency of their offspring. Fertility records (conception to 1st postpartum AI or TC1; and calving to conception interval or IPC) milk production standardized for 305 days of lactation (average production of 12.335 kg/lactation) in a total of 56.132 primiparous Holstein cows (offspring), from 226 commercial dairy herds located in the Central California Valley (USA), were used in this analysis. Information regarding IPC of the dam as well as the dam's parity (dam being a nulliparous heifer N=25750 or a lactating cow N=30384) were paired with the reproductive performance of their offspring, which were cows at their first lactation. Data were analyzed with the proc HPMIXED of the SAS software (version 9.3). When we divided the dam's IPC in classes (<50 d, 51-100 d, 101-150 d, 151-200 d, 201-300 d, and >300d), we observed that both TC1 and IPC of the offspring during their first lactation was affected (P<0.01), respectively: Dam's with IPC < 50 d=38.4% and 113d; 51-100 d=36.9% and 120d; 101-150 d=35.1% and 121d; 151-200 d=35.8% and 123d; 201-300 d=34.7% and 124d; > 300 d=31.5% and 127d. Unexpectedly, the uterine environment of the dam did not (P=0.15) affect future fertility of their offspring and daughters from heifers had TC1=37.3% and IPC=119 d and daughters from lactating cows had TC1=36.0% and IPC=121 d. These results suggest that dams with poor reproductive efficiency will tend to produce daughters with compromised fertility; however, lactation status of the dam did not seem to affect fertility in their offspring.

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A113 FTAI, FTET and AI

Artificial insemination and FTAI in dairy herds in Acre state: a case report

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EMBRAPA.

Keywords: artificial insemination, dairy cattle, FTAI.

The state of Acre has one of the lowest daily average milk production per cow in Brazil reflecting a poorly technified production. This fact has produced a great requirement for genetic improvement of the herd so that producers can increase their productive efficiency. Artificial insemination (AI) is a widespread technique and aims the genetic improvement using semen of genetically superior bulls. Recently, to overcome the main difficulty of this technique, which is the estrus detection, AI has been used in fixed time (FTAI). However, both techniques must be implemented appropriately to avoid reduction in reproductive efficiency. The objective of this study was to report results of pregnancy rate obtained after the introduction of AI, with estrus detection, associated with FTAI in dairy herds. The AI program was started from a theoretical-practical course for family dairy farmers. Two farms located in Feijó city, in the state of AC, were enrolled. Pregnancy diagnosis was performed using ultrasonography (Mindray DP 2200) and herd data was collected. Pregnancy rates were evaluated before (natural mating) and after implementation of AI + FTAI. Pregnancy diagnosis was performed in all animals before the program implementation [first assessment (A1)] and 12 months later [second assessment (A2)]. At A1, a pregnancy rate of 58% (n= 48/83) was observed in the farm 01 for animals in reproductive age, using FTAI + AI, and natural mating during a specified period of 2 months. After A1, 9 animals were culled due to low production and advanced age. In the farm 02, a pregnancy rate of 50% (n= 13/26) was observed for animals in reproductive age, using FTAI and natural mating during a specified period of 2 months. There was no culling in farm 02. At A2, in the farm 01, a pregnancy rate of 77% (n=57/74) was observed, in which 82.5% of the pregnancies resulted from AI (conception rate of 48%) or FTAI (conception rate of 38%). In the farm 02, a pregnancy rate of 73% (n=19/26) was observed, in which 93% of the pregnancies resulted from FTAI (conception rate of 50%). In conclusion, the use of AI associated to FTAI increased the pregnancy rates in dairy herds, with satisfactory numbers of pregnancies.

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A114 FTAI, FTET and AI

Intensification of reproductive management in a dairy herd

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Keywords: dairy cattle, FTAI, reproductive management.

In order to evaluate the impact of the intensive use of fixed time artificial insemination (FTAI) on reproductive efficiency in a dairy herd in São Paulo State, Brazil, an analysis of 4512 artificial inseminations (AI; 1688 in primiparous and 2824 in multiparous cows) was performed between 2009 and 2014. These data were from 320 lactating dairy cows, managed in a free stall system with average production of 35.4±9.4 kg of milk/d. For analysis, two groups were established based on the reproductive management strategy: Non-Intensive (N-INT; 2009-2011), where cows were treated with two applications of PGF2α at 40±7 and 54±7 days in milk (DIM), and on 73±7 DIM cows were subjected to FTAI; and Intensive (INT, 2012-2013), in which cows received PGF2α (at 40±3 DIM), and after 14 d (54±3 DIM) were subjected to the FTAI. In both groups, after the voluntary waiting period (VWP; 40 DIM), cows observed in estrus were inseminated. For the N-INT group pregnancy diagnosis was conducted every 14 d, and for the INT, it was done every 7 d. Data were analyzed using the Freq, GLIMMIX and Lifetest procedures of SAS. The percentage of inseminations performed by FTAI was higher (P<0.001) in the intensive management period (INT: 56.9% [1474/2592] vs N-INT: 29.1% [559/1920]). In the survival analysis, N-INT group received the first AI (P<0.01) and became pregnant (P<0.01) later than the INT group. At 70 DIM, 65.4% (394/572) of N-INT cows had not been inseminated yet, while in the INT group only 35.2% (314/892). Furthermore, it was seen that 34.2% (184/538) and 45.4% (408/899) of cows became pregnant at 103 DIM (three 21-d cycles after the VWP) for N-INT and INT groups, respectively. The N-INT group had lower P/AI at 31 d post insemination (27.9% [539/1920] vs. 37.1% [903/2592]; P<0.01) and at 59 d (23.8% [463/1920] vs. 32.4% [777/2592]; P<0.01). However, there was no difference in pregnancy loss (13.6% [76/539] vs. 13.6% [126/903]; P>0.10). When we analyzed the interaction between groups and AI number, there was no effect (P=0.17) on P/AI at 31 d (23.6% [160/577], 28.4% [379/1343], 34.2% [341/898], and 34.8% [562/1694] for N-INT 1stAI, N-INT ReAI, INT 1stAI, INT ReAI, respectively), however on 59 d there was effect (22 6% [133/577], 25.0% [330/1343], 34.3% [302/898], and 30.5% [475/1694] for N-INT 1stAI, N-INT ReAI, INT 1stAI and INT ReAI, respectively; P=0.04). Therefore, the intensification of the reproductive management increased service rate to first AI, and consequently decreased the calving to conception interval. Furthermore, it improved fertility of the first postpartum AI.

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A115 FTAI, FTET and AI

Improvement of pregnancy rate by using the 6-days J-synch protocol in recipient cows transferred with *in vitro* produced embryos

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Keywords: corpus luteum, embryo, ovary.

Proestrus length affects the ability of the follicle to growth and achieve ovulation, so pregnancy rate could be improved if proestrus is prolonged. Taking this into account, a new protocol for FTAI using estradiol and progesterone has been proposed named as J-Synch. In this new treatment the removal of the device is advanced to Day 6 instead Day 7 or 8, and GnRH is given 72 h later to induce ovulation instead of ECP at device removal. The objective of this study was to determine pregnancy rate in recipient females obtained with the conventional 7 d vs. the new 6 d progesterone treatment. The experiment was performed in five replicates using 945 cycling Hereford crossbreed recipients with body condition score >2.5 (1 to 5 scale). All the females received an intravaginal device (0.5 g progesterone, DIB, Syntex, Argentina) plus 2 mg EB i.m. (Syntex) (Day 0). In the 7-day progesterone + ECP treatment group (n=481) the DIB was removed on Day 7 in the morning and 500 µg cloprostenol (Ciclase DL, Syntex), 400 IU eCG (Novormon, Syntex), and 0.5 mg ECP (Cipiosyn, Syntex) was administrated i.m.. In the experimental J-Synch group (n=464), DIB was removed on Day 6 at time of cloprostenol and eCG was given i.m.. For this group the females received one dose of GnRH analogue (100 µg gonadorelin acetate, Gonasyn Gdr, Syntex) given 72 h after device removal. Fixed time embryo transfer (FTET) was performed on Day 16 and 17 with blastocysts produced by in vitro fertilization using sexed semen. Pregnancy rate was determined by ultrasonography 40-45 days after embryo transfer. Data were analyzed using logistic regression. Pregnancy rate was higher for the 6-days treatment (49.3%, 229/464) than conventional treatment (40.9%, 197/481; P<0.05). No effect for the day of the embryo transfer was found (FTET on Day 16: 45.9%, 249/543; FTET on Day 17: 44.3%, 178/402; P=NS). In conclusion, the extension of the period between progesterone fall and the treatment with the inducer of ovulation reached by the advancement of DIB removal on Day 6 and GnRH administration on Day 9 (i.e. J-Synch protocol) could improve pregnancy rate in recipient cows.

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A116 FTAI, FTET and AI

Simultaneous thawing of ten semen straws reduces conception rates of beef cows submitted to TAI

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Keywords: Nelore, semen thawing, TAI.

The aim of this study was to evaluate the effects of different methods of semen thawing on conception rate of beef cows undergoing artificial insemination at fixed time (TAI). A total of 1174 cows (1130 lactating and 44 non-lactating) with an average body condition score (BCS) 3.00±0.01, from seven commercial farms, received at the beginning of treatment (Day 0), a progesterone release device (P4; DIB®; MSD Animal Health) combined with 2.0 mg estradiol benzoate IM (BE; Gonadiol®, MSD Animal Health). Eight days after (Day 8), the device was removed and cows received simultaneously 1 mg IM of estradiol cypionate (ECP Zoetis Animal Health), 530 mg IM sodium cloprostenol (Ciosin®, MSD Animal Health) and 300 IU IM of equine chorionic gonadotropin (Folligon®, MSD Animal Health). On Day 10, all cows were inseminated with semen straws (0.25 ml) thawed by two different methods, as follows: 1) Conventional (n=575), in which 10 semen straws were thawed simultaneously at 36°C using automatic defroster (CITO Warm Water Thaw; DSP-0063/AC)); or 2) Paused (n=599) in which only three straws were thawed simultaneously using the same temperature and automatic defroster to defrosting. After 30 seconds of thawing, straws were used sequentially (approximately one TAI per minute). Pregnancy diagnosis was done by transrectal ultrasonography (5.0 MHz, Aloka and Mindrey) 30 days after TAI. Statistical analysis was performed using SAS PROC GLIMMIX (9.3 SAS ® Institute, Inc., Cary, NC, USA, 2003). The Paused method resulted in a greater conception rate (50.7%) compared to the conventional method (42.3%; P=0.01). In conclusion, simultaneous thawing of three straws promotes better results than the simultaneous defrosting of 10 straws in lactating beef cows inseminated at fixed time.

Acknowledgments: Firmasa Tecnologia para Pecuária, Grupo Piveta, Camilha and Carolina Agulhon (Romaria Farm).

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A117 FTAI, FTET and AI

Does artificial insemination procedure influences the cortisol plasma concentrations in cattle?

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Keywords: artificial insemination, cortisol, radioimmunoassay.

The goal of this study was to determine whether the artificial insemination procedure (AI) is able to influence cortisol plasma concentration, due to stress that animals are subjected, as well as to correlate cortisol concentration and uterine hemodynamics data assessed by ultrasound Doppler in postpartum Nellore cows. Eighteen females were divided into two groups: inseminated (AIG, n=9) and non-inseminated (CG, n=9). All animals underwent estrus and ovulation synchronization; however, only AIG animals had semen deposited in the uterine body. All animals were evaluated for uterine hemodynamics using a Doppler ultrasound in the color mode, to assess vascularization score of the uterine horns (VS) and in the spectral mode, to evaluate uterine artery resistance index (RI) in five moments: 30 hours before AI, 4, 24, 48 and 168 hours after AI. Simultaneously, blood samples were collected from the jugular vein from all animals. Plasma was evaluated with a specific radioimmunoassay kit for determination of cortisol concentration. Cortisol levels are shown in nmol/L. Data were analyzed using PROC MIXED to detect differences between the groups and the PROC CORR of SAS was used to verify the correlation between the variables. Statistical significance was considered when $P<0.05$. There was no difference in cortisol concentrations between groups (AIG=78.70±8.93, CG=68.71±5.30, P=0.33). Regarding uterine hemodynamics, AIG had lower RI values (AIG=0.67±0.01; CG=0.73±0.02; $P<0.05$), indicating increased vascularization, however, no difference was observed between the groups regarding VS (AIG=1.81±0.08; CG=1.75±0.05; $P>0.05$). There was also no correlation between cortisol concentration and VS (R=-0.00527, P=0.96) nor between cortisol concentration and RI (R=-0.00577, P=0.95). In these circumstances, and considering the limited amount of animals (n=18), we could not observe differences in plasma cortisol concentration between animals that were inseminated or not, and there was no correlation between plasma cortisol concentration and uterine hemodynamics assessed by Doppler ultrasound.

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A118 FTAI, FTET and AI

Protocols for synchronization of follicular wave emergence and ovulation and the use of eCG can acelerate postpartum stablishment of pregnancy in primiparous Nelore (*Bos indicus*) cows subjected to natural mating

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Keywords: anestrous, bull, cyclicity induction.

We studied the effect of using protocols for synchronization of follicular wave emergence and ovulation associated or not to eCG on time to conception during the breeding season (BS) of primiparous Nelore cows kept on pasture with bulls. The study took place at Vera Cruz IV Farm, Barra do Garças – MT. At D0, 350 primiparous cows (30-50 DPP) were evaluated by ultrasonography (Mindray DP2200VET). Only those with complete uterine regression were randomized (taking into account their BCS) to one of three groups: without treatment (control), treated (protocol without eCG), or treated+eCG (protocol with eCG). Control cows received no previous treatment before bull mating, while treated cows received similar protocol except for the use of eCG (Ecegon®;Biogénesis Bagó, Argentina). The protocol consisted on the insertion of a 1g P4 device (Cronipres® Mono Dose, Biogénesis Bagó, Brazil) and 2mg estradiol benzoate (Bioestrogen®, Biogénesis Bagó, Brazil) IM on D0. On D9, device was removed and 1mg estradiol cypionate (Croni-Cip®, Biogénesis Bagó, Argentina) was given IM. Additional 300 IU of eCG was given only to treated+eCG cows. All cows were kept at the same pasture and exposed to bull mating on D9. The proportion bull:cow was 1:10 within the first 10d and 1:20 within the other 110d of the BS. Ultrasound exams were done at every 40d, registering gestational age to predict the quantity of new gestations every 21d cycle (C1, C2, C3, C4 and C5), starting at bull exposure (C1). Data was analyzed by PROC GLIMMIX, SAS. Control cows had lower conception at C1 (5.7%^{c}, 7/123) than treated (30.4%^{b}, 35/115) and treated+eCG (51.8%^{a}, 58/112; P=0.001). One cycle later (C2) there was an increase on conception of all groups, but the difference between them was kept: 17.1%^{c} (21/123), 42.6%^{b} (49/115), 58.9%^{a} (66/112); P=0.001. Similar effect occurred at C3 [27.6% (34/123)^{c}, 52.2% (60/115)^{b} and 70.4% (79/112)^{a}; P=0.001] and C4 [42.3% (52/123)^{c}, 58.3% (67/115)^{b} and 74.1% (83/112)^{a}; P=0.001]. Only at C5 control cows got closer to treated, but treated+eCG kept higher conception: 65.0% (80/123)^{b}, 68.7% (79/115)^{ab} and 82.1% (92/112)^{a}; P=0.01. Thus, it was shown that (1) PP acyclicity is an important villain of reproductive efficiency of primiparous cows kept on pasture and that (2) treatment with TAI-like protocols is crucial to improve and accelerate PP conception. Also, the use of eCG potentially increased PP conception by allowing more than half of cows to conceive at the first PP service, which is higher than 5.7% and 30.4% found for control and treated cows without eCG. The velocity of PP conception is crucial to achieve 12m interval between parturitions and maximum herd reproductive efficiency. Thus, the use of TAI-like protocols associated with eCG can anticipate PP cyclicity and is a potential tool to improve conception of primiparous cows exposed to bulls, with impact until the end of the BS.

Credits: Farm Vera Cruz IV, FAPESP 2012/07510-1, CNPq 486089/2013-4.

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A119 FTAI, FTET and AI

Loss of active ingredient in the process of reuse of intravaginal devices of progesterone

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Keywords: concentration, devices, progesterone.

The process of reutilization of intravaginal devices (VIP), although not indicated by manufacturers, is commonly used in Brazil. However, more information on cleaning these devices for reutilization is much needed (Neri et al., Arq. Bras. Med. Vet. Zootec., v.67, n.2, p.405-410, 2015) to elucidate possible problems during the cleaning process and disinfestation of VIP aiming its reutilization. Usually, there is a great concern in regards to the loss of progesterone in this cleansing process. Thus, the current study is justified due to lack of information on this subject matter. This study then aimed to quantify the amount of P4 lost during cleaning of VIP when left resting in solution with or without detergent. We used ten new silicon-based VIP, loaded with 1g of P4 (Primer® - AgenerUniao – Brazil). IVP devices were placed in a clean bucket filled with 5 liters of filtered water with or without addiction of 1% detergent solution. The implants were fully submerged in water. Immediately before each collection, the pre-set times samples of 0,1ml were collected with automatic pipette at times 0 (before put the VIP), 30, 60 and 120 minutes after putting the VIP. Before collect the samples containers were homogenized. The samples collected were diluted 100x to adjust the range of measurement Kit RIA (0.5 to 40ng/ml). In each container with 0.1ml of original sample were added 9,9ml of other solution. About the final total sample, 1ml was collected and placed on an eppendorf and frozen at -20^{o}C for later evaluation of P4 by RIA (RIA IM 1188, Immunotech Inc., Prague, CzechRepublic). Each implant had originally 1g P4. The amount of P4 lost was determined by the concentration/ml x 5.000 (solution volume). As the dosage of P4 was expressed in ng/ml the loss percentage was calculated by the following formula: (TotP4Sol/109)*100=TotP4Sol/107. Average of P4 was accessed during periods and compared by ANOVA and TUKEY test. In containers without detergent the P4 averages differed among themselves on evaluated periods (26.6+8.7^{a}; 35.0+9.2^{b} and 40.6+10.4^{b} ng/mL for measured times of 30, 60 and 120 minutes for P>0.05),. Similarly, detergent containers had an increase of [P4] overtime (42.2+11.2^{a}; 57.0+13.5^{b} and 69.2+15.1cng/mL for measured times of 30, 60 and 120 minutes respectively). For the same time of sample collections the inclusion of detergent led to an increase in P4 in the solution (P<0.05). Despite the P4 loss, the percentage of P4 losses were negligible if we consider the total amount of P4 in the device. For the solution having no detergent the average of P4 loss was (1.33%, 1.75% and 2.03%, at 30, 60 and 120 minutes). The addiction of detergent in the solution resulted in average P4 percentage loss of (2.36%, 2.85% and 3.46% for 30, 60 and 120 minutes in solution). In conclusion, although there were losses, these P4 losses are not significant when the implants are left in solution with or without detergent for up to 120 minutes.

Thanks: Fapemig, Capes and CNPq.

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A120 FTAI, FTET and AI

Embryo production of Santa Inês ewes superovulated with FSH

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Keywords: embryo, sheep, superovulation.

In this study we evaluated the effect of administration of decreasing doses of FSH 12/12 or 24/24 on superovulatory response and embryo production of Santa Inês ewes. We used 34 ewes divided into two treatments. For SOV protocol synchronization of estrus was obtained by placing vaginal CIDR with 0.3 g of P4 for 14 days, with device exchange on the seventh day, this was also applied 37.5 ug of D - cloprostenol. Ovarian stimulation was performed with total dose of 133mg of FSH. In treatment 1 (T1; n=17) donors received eight applications in decreasing doses every 12 hours and treatment 2 (T2; n=17) consisted of 4 applications of FSH in decreasing doses every 24 hours, started 48 hours before removal CIDR in both treatments. Twelve hours after CIDR removal ewes received 25 g of lecirelin as ovulation inducer; AI with frozen semen by laparoscopy was performed 12 and 22 hours after lecirelin and embryo collection through hemilaparoscopy occurred five days (d5) after AI. Ultrasonographic evaluation was performed in 50% of donors. The statistical analysis was performed with the program SAEG 9.1 applying the parametric and non-parametric tests. The average number of collected structures (T1=3.47, T2=2.55) were similar between treatments (P> 0.05). Viable embryos (T1=2.40, T2=0.82), freezable embryos (T1=2.07, T2=0.82) and the average number of LC (T1=7.47, T2=5.73) in the group of ewes superovulated with decreasing doses of FSH distributed in eight applications 12/12 was higher than the group of ewes who received four applications of FSH at 24/24 (P<0.05). In addition, the superovulatory response was similar (P>0.05) between donors T1 (88.24 %) and T2 (64.70%). The results suggest that the FSH regimen of eight-doses at 12-hour intervals is a better option for in Santa Inês ewes.

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A121 FTAI, FTET and AI

Reducing beef cows handling to perform timed artificial insemination with sex-sorted sperm

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Keywords: estradiol esters, sexed semen, TAI.

This study aimed to compare different ovulation inductors (estradiol benzoate or cypionate) during timed artificial insemination (TAI) protocols with sex-sorted sperm to reduce cattle handling (3 vs. 4 managements) and to evaluate the reproductive efficiency of suckled beef cows. At a random stage of the estrous cycle (Day 0), a total of 591 Nelore (Bos indicus) multiparous cows with BCS 2.45±0.02 received a new insert of progesterone (P4 device; CIDR®, Zoetis) plus 2 mg IM estradiol benzoate (EB; Gonadiol®, Zoetis). At P4 device removal (Day 8), cows received 25 mg IM of Dinoprost (Lutalyse®; Zoetis) and 300 IU of eCG (Novormon®; Zoetis) and were randomly allocated into one of four experimental groups in a factorial 2 x 2 design: 1) 1 mg of estradiol cypionate (EC; E.C.P®, Zoetis) at P4 device removal and TAI with non sex-sorted sperm (EC-0h-Conv; n=148); 2) the same treatment of EC-0h-Conv, inseminated with sex-sorted sperm (EC-0h-Sex; n=149); 3) 2 mg of EB 24 hours after P4 device removal and TAI with non sex-sorted sperm (EB-24h-Conv; n=145); and 4) the same treatment of EB-24-Conv, inseminated with sex-sorted sperm (EB-24h-Sex; n=149). All TAIs were performed by three experienced AI technicians, 60 hours after P4 device removal using the same batch of three different sires (A, B and C). Cows were examined for pregnancy by transrectal ultrasonography (5.0 MHz linear transrectal transducer, Chison D600VET, Eletromedicina, Brazil) 30 d after TAI. Statistical analysis was performed using the PROC GLIMMIX procedure of SAS (SAS® 9.3). The BCS was considered as a covariate and AI technicians included in the model as random effect. There was no interaction (P=0.07) between different ovulation inductors and semen type on pregnancy per AI (P/AI) which was, as follows: EC-0h-Conv=68.2% (101/148); EC-0h-Sex=51.0% (76/149); EB-24h-Conv=52.4% (76/145); and EB-24h-Sex=49.0% (73/149). However, EC promoted greater P/AI in comparison to EB [EC=59.6% (177/297) vs. EB=50.7% (149/294); P=0.03)] and non sex-sorted sperm resulted in greater P/AI compared to sex-sorted sperm [Non sex-sorted=60.4% (177/293) vs. Sex-sorted=50.0% (149/298); P=0.007]. Furthermore, there was a difference according to sires on P/AI, regardless the ovulation inductors or type of semen used [sex-sorted sperm: Sire A=39.8% (39/98); Sire B=50.0% (51/102) and Sire C=60.2% (n =59/98). Non sex-sorted sperm: Sire A=54.5% (55/101); Sire B=62.1% (64/103) and Sire C=65.2% (58/89); P=0.01]. Thus, non sex-sorted sperm resulted in greater P/AI and different sires resulted in different P/AI. Also, EC resulted in greater P/AI in comparison to EB, allowing reduced handling of suckled beef cows to be timed inseminated with sex-sorted sperm without compromising P/AI.

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A122 FTAI, FTET and AI

Relationship between population of antral follicles and uterine diameter in Nelore heifers

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Keywords: *Bos indicus*, follicular population, puberty.

The relationship between the number of follicles and hormone levels were shown to possible parameters to represent the reproductive potential *Bos taurus* females (Ireland et al., 2011, Reprod. Fertil. Dev. 23: 1-14). The aim of this study was to compare the relationship between the population of antral follicles and uterine diameter. Three hundred and thirty six Nelore heifers, 25.0±3.2 months old (BCS: 3.0 to 3.5, range 1-5) managed in Brachiaria brizantha and mineral supplementation containing 9% phosphorus were used during the month of October 2014. The animals were submitted to two transrectal ultrasonography exams (7.5 MHz, Mindray, China) with 10 days interval, for evaluation of cyclicity, viewing CL, counting all the visualized antral follicles (AFC) and measurements of uterine diameter, after obtaining three transversal evaluation and the average dimensions. The results were submitted to analysis of variance and correlation with significance level of 5% and heifers were classified as low (12.5±2.7; n=73), intermediate (21.7±2.4; n=86) AFCand high (35.0±7.7; n=177) (P=0.001). The uterine diameter was similar (P>0.05) for high (11.0±1.9 mm), intermediate (10.8±2.0 mm) and low AFC (10.3±2.7 mm). Prepubertal (n=280), pubertalheifers (n=56) had difference (P=0.03) in values for AFC, resulting in 27.3±11.1 vs. 23.7±8.0 follicles, respectively. The uterine diameter was greater (P=0.001) in pubertal (12.2±2.6 mm) than in prepubertal heifers (10.6±1.9 mm). The AFC was positively correlated with the size of the uterus (CR=0.11; P=0.03), however there was a positive correlation only in prepubertal heifers (CR=0.17; P=0.003) (pubertal: CR=- 0.05; P=0.6). The results suggest that follicular population has been possible to influence the uterine development prepubertal Nelore heifers.

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A123 FTAI, FTET and AI

Resynchronization protocols improve reproductive efficiency of suckled beef cows subjected to a breeding season during autumn-winter

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Keywords: bovine, breading season, TAI.

The present study evaluated the impact of resynchronization (RE) after the first timed artificial insemination (TAI) in suckled beef cows (n=960; Angus and Brangus) submitted to breeding season (BS) during the autumn-winter. Females were subjected to one of four reproductive managements: 1) only natural mating (MN) during 90 days of the BS (n=266); 2) TAI at the first day of the BS (Day 0) followed MN until the end of the BS (TAI+MN; n=200); 3) TAI followed RE 30 days after the 1st TAI and MN until the end BS (TAI+RE30+MN; n=245) or; 4) TAI followed RE 22 days after the 1st TAI and MN until the end EM (TAI+RE22+MN; n=249). Cows were randomly assigned according to the calving date into the breeding groups and allocated in winter pastures composed of Avena sativa, Lolium multiflorum and Trifolium reprens. The TAI protocols were similarly for TAI and RE. The treatment consisted of the insertion of a progesterone (P4) device (DIB®, MSD Animal Health) plus 2 mg of estradiol benzoate (EB, GONADIOL®, MSD Animal Health). Eight days later, the P4 devices were removed and were administered 500 mg of sodium cloprostenol (Ciosin ® MSD Animal Health), 1 mg of estradiol cypionate (ECP®, Zoetis) and 400 IU of equine chorionic gonadotropin (Folligon®, MSD Animal Health). The TAI was performed 48 hours after P4 device removal. Cows from MN group were exposed to bull during the entire 90 days BS, while the other groups, the bulls were introduced 10 days after the last TAI, using the proportion of one bull for each 20 cows. The pregnancy diagnosis were performed 30 (diagnosis of MN or pregnancy after the 1st TAI), 70 (diagnosis of MN or pregnancy after the 2nd TAI) and 120 (final diagnosis, 30 days after the end of BS) days after the onset of BS. Statistical analysis was performed by logistic regression using SAS PROC GLIMMIX. The effect of cow was considered as a random effect. At 30 days of BS, females undergoing a MN (3.0%) had lower pregnancy rate (P<0.001) compared to other groups (TAI+MN=40.0%; TAI+RE30+MN= 40.0%; TAI+RE22+MN=39.8%). At 70 days of BS, the groups receiving RE [TAI+RE30+MN (69.4%) and TAI+RE22+MN (66.3%)] obtained greater pregnancy rate (P<0.001) than the other groups [MN (16.9%) and TAI + MN (48.0%)]. However, the TAI+MN group had greater pregnancy rate than the MN group (P<0.001). The pregnancy rate at the end of BS was greater in cow receiving RE [TAI+RE30+MN (83.7%a) or TAI+RE22+MN (81.5%a)] than the MN (45.1%c) or TAI+MN (71.0%b). Still, females subjected to TAI+MN obtained greater pregnancy rate at end of the BS than those submitted to only MN (P <0.001). Thus, the use of resynchronization programs, RE30 or RE22, increase the proportion of pregnancy from IA and the reproductive efficiency of *Bos taurus* cows submitted to BS during the autumn-winter.

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A124 FTAI, FTET and AI

Supplementation with melengestrol acetate (MGA) post TAI improves fertility in suckled Nelore cows

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[1]UNESP; [2]ZOETIS.

Keywords: MGA, progestogen, TAI.

The objective of this study was to evaluate if oral supplementation of Melengestrol Acetate (MGA®, Zoetis, SP, Brazil) after TAI and the supplementation period increases pregnancy rate in Nelore cows submitted to TAI protocols with different gonadotropin stimulation (Calf removal (CR) or eCG). A total of 2301 multiparous suckled Nelore cows was enrolled to the experiment, distributed in 24 lots with similarly days post partum, and access to pasture of *Braquiaria* and water *ad libitum*. All cows were synchronized with the protocol: 2.0 mg of Estradiol Benzoate im (2.0 ml of Gonadiol®, Zoetis, SP, Brazil) + intravaginal progesterone device (CIDR®, Zoetis, SP, Brazil) in d-11; 12.5 mg of PGF2α im (2.5 ml of Lutalyse®, Zoetis, SP, Brazil) in d-4; Removal of CIDR® + 0.5 mg of Estradiol Cypionate im (0.3 ml of ECP®, Zoetis, SP, Brazil) in d-2; AI in d0. In d-2 15 lots (Farm 01) received 300 IU of eCG im (1.5 ml of Novormon®, Zoetis, SP, Brazil) and 9 lots (Farm 02) received CR. After insemination each lot of animals were randomly distributed in three treatments: Control- Animals did not receive MGA; MGA 6- MGA supplementation during 6 days, from day 13 to day 18 after TAI; MGA 14- MGA supplementation during 14 days, from day 5 to day 18 after TAI. Melengestrol Acetate dose utilized was 0.5 mg/ day (2.28 g of MGA®/day) supplied with mineral salt every day. Mineral salt intake was evaluated for 3 days before the start of supplementation. Pregnancy diagnosis was performed by ultrasound 30 days after insemination. Pregnancy per AI (P/AI) was determined by dividing the number of pregnant cows by the number of inseminated cows. Was used the PROC GLIMMIX of SAS program for data analysis, random variables were included in the model and withdrawals (criterion of Wald) when P>0.2. Two contrasts were used to determine the effect of MGA supplementation [Control vs. MGA (MGA 6 + MGA 14)] and to determine the effect of MGA supplementation duration (MGA 6 vs. MGA 14). Significant differences were considered when P≤0.05. There was no interaction between BCS or stimulus (CR and eCG) on pregnancy rate. Cows supplemented with MGA had higher P/AI [62.7% (983/1568)] compared to control group [56.2% (412/733)] independent of gonadotropin stimulation. The duration of supplementation, independent of gonadotropin stimulation, did not change pregnancy rates [MGA 6: 61.9% (520/840) vs. MGA 14: 63.6% (463/728)]. We concluded that MGA supplementation post TAI increased pregnancy rates in suckled Nelore cows, independent of gonadotropin stimulation and duration of supplementation.

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A125 FTAI, FTET and AI

Supplementation with sunflower seed alters the endometrial lipid composition in beef cows

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[1]UNESP; [2]USP.

Keywords: linoleic acid, lipids, PGF2α.

Compounds that are rich in linoleic acid, such as sunflower seeds, provide lipid changes in the endometrium, and may be involved in the ability of PGF2α biosynthesis. Previous studies (Cordeiro et al. 2015, Theriogenology, 83, 9, p. 1461-1468) observed that the conception rate increased in Nelore cows supplemented with sunflower seed for 22 days from the TAI (66.7 % vs 47.8 %) and in crossbred heifers submitted to TET (55.7 % vs. 36.9 %). We aimed to test the hypothesis that supplementation with sunflower seed promotes endometrial changes in lipid composition. Thus, we compared the composition of fatty acids in endometrial tissue in cows supplemented or not with sunflower seed. Nelore (n=30) cows received an intravaginal device containing progesterone (1g; DIB, Syntex Biochemistry & Pharmaceutical, Buenos Aires, Argentina) associated with an im injection of estradiol benzoate (2mg; Benzoate HC, Hertape Calier Animal Health, Juatuba, Brazil). The devices were removed after eight days, when cows were treated im with cloprostenol sodium (2mg; Sincrocio®, Ourofino Animal Health, Ribeirão Preto, Brazil), estradiol cypionate (0.5 mg; ECP®, Zoetis, São Paulo, Brazil) and eCG (300IU; Folligon®, MSD Animal Health, Madison, USA). Two days after removal of the device females were assigned into six groups to receive 1.7 kg/cow/day of 40% soybean meal, 44% crude protein (CP) + 60% sunflower seed for 6 (n=4), 14 (n=5) and 22 days (n=6), or 53% soybean meal, 44% CP + 47% corn for 6 (n=4), 14 (n=5) and 22 days (n=6). Both diets were formulated with 72% TDN and 24% CP. Females were slaughtered 24 hours after the end of supplementation and endometrial tissue was isolated and stored at -196^{o}C. The fatty acids in endometrial tissue were assessed by gas chromatography. Data were analyzed by SAS Proc GLIMMIX. The fatty acid profile (54 compounds) was analyzed and 43 fatty acids were present in the endometrial tissue. The lacking fatty acids in endometrial tissue were C4:0, C11:0, C12:1, C: 13:0, C13:0 iso, C13:0 anteiso, C14:0 iso, C15:1, C18:1 T16, C18: 2 C12 T10 and C21:0. The fatty acids that showed a higher percentage compared to Control group were C18:1 T10-T11-T12 and C10:1. The fatty acids that showed low percentage compared to the Control group were C15:0 iso, C20:5, C20:3N3, C23:0, C24:0 and C22:5. In conclusion, the supplementation with sunflower seed promotes changes in the endometrial lipids. More studies are needed to examine the possibility of reducing embryonic mortality with such supplementation.

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A126 FTAI, FTET and AI

Conception rate to a TAI protocol in Nelore (*Bos indicus*) cows submitted to three or four managements using Sincrogest® or CIDR®

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Keywords: fertility, management, progesterone.

The aim of this study was to evaluate the conception rate in multiparous Nelore (*Bos indicus*) cows using two intravaginal progesterone (P4) devices (Sincrogest®; 1.0 g of P4 or CIDR®; 1.9g P4) during the TAI protocol 3 (Day 0, Day 8 and Day 10) or 4 (Day 0, Day 7, Day 9 and Day 11) managements. A total of 372 cows Nelore multiparous, from a herd located in Aquidauana/MS, was homogeneously distributed according to body condition score (mean of 2.90±0.02) and days postpartum (mean 38 8±0.3 days) at the beginning of the TAI protocol. At initiation of the TAI protocol (D0) the animals were treated with 2.0 mg EB IM (Sincrodiol®, Ouro Fino, Brazil) and insertion of the intravaginal device release of P4 (SINCROGEST® or CIDR®). To avoid additional managements from this point onwards, the cows were managed separately according to the type of protocol (3 vs. 4 managements). In cows receiving the protocol with 3 managements, withdrawal of P4 device was performed on D8 (Sincrogest® or CIDR®) associated to the treatment with 0.530 mg of sodium cloprostenol IM (Sincrocio®, OuroFino, Brazil), 300 IU IM of equine chorionic gonadotropin (eCG; SincroeCG®, Ouro Fino, Brazil) and 1.0 mg IM of estradiol cypionate (ECP®, Pfizer, Sao Paulo, Brazil). The TAI was done on Day 10 (a.m.). For cows enrolled in the protocol with 4 managements, 0.530 mg of sodium cloprostenol (Sincrocio®, Ouro Fino, Brazil) was administered on D7. The, on Day 9, P4 devices (Sincrogest or CIDR) were removed associated with 300IU of equine chorionic gonadotropin (eCG; SincroeCG®, Ouro Fino, Brazil) and 1.0 mg of estradiol cypionate (ECP®, Pfizer, São Paulo, Brazil). The TAI was performed on Day 11 (a.m.). After TAI, the groups of 3 and 4 managements were again regrouped until the time of pregnancy diagnosis performed by ultrasonography (Mindray, DP2200vet, São Paulo, Brazil) 30 days after TAI. Data were analyzed using the SAS procedure GLIMMIX 9.3. No interaction was observed between P4 device and the number of managements on conception rate [Sincrogest®*3managements = 50.56% (45/89); CIDR®*3managements = 54.90% (56/102); Sincrogest®*4managements=52.63% (50/95); CIDR®*4managements = 44.2% (38/86); P=0.41). Likewise, there were no differences in conception rate between the groups treated with different P4 devices [Sincrogest® = 51.63% (95/184) vs. CIDR® = 50.00% (94/188); P=0.46], as well as number of managements, [3 managements = 52.88% (101/191); 4 managements = 48.62% (88/101); P=0.43]. Thus, it is possible to obtain similar conception rates to TAI in lactating zebu cows treated with Sincrogest® or CIDR®, regardless of number of managements employed (3 or 4 managements) during the TAI protocol.

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A127 FTAI, FTET and AI

Pregnancy rate in Nellore females supplemented with oral progestogen after TAI

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Keywords: cyclicity, fertility, progesterone.

This study aimed to determine the impact of supplementation with oral progestogen after TAI on the pregnancy rate of cyclic and anovular Nellore cows. Two hundred and eighty three cows with 35 to 68 days postpartum and average body condition score of 3.35 (from 1 to 5) were subjected to two clinical and gynecological examinations (ten-day interval) with the aid of transrectal ultrasonography using linear transducer frequency 6.0MHz (Pie-Medical, Falco 100, São Paulo, Brazil) to estimate the reproductive status, being considered in anovular the cows without the presence of corpus luteum (CL) at both evaluations. Subsequently, all cows were subjected to the following synchronization protocol: at a random day of the estrous cycle (Day 0) they received an intravaginal progesterone device (P4, CIDR®, Zoetis, São Paulo, Brazil) associated with 2 mg of estradiol benzoate by intramuscular (IM) (GONADIOL®, Zoetis, São Paulo, Brazil). On day 8 (D8) the intravaginal progesterone device was removed and 25 mg of Dinoprost tromethamine was administered IM (Lutalyse®, Zoetis, São Paulo, Brazil), 1 mg of estradiol cypionate IM (ECP®, Zoetis, São Paulo, Brazil) and 300IU of eCG IM (NOVORMON®, Zoetis, São Paulo, Brazil). In Day 10 (D10), all cows were inseminated. On the 13th day after TAI, the cows were evenly distributed according to the prior cyclicality at the beginning of the protocol into two groups: MGA (n=154) cows supplemented with melengestrol acetate (MGA PREMIX®, Zoetis, São Paulo , Brazil) at a dose of 2.28 g per cow/day given in the trough with the mineral salt for a period of 6 days (between the 13th and 18th day after TAI) and NoMGA (n=129) cows received in the trough just the mineral salt without supplementation with MGA. Pregnancy diagnosis was done by transrectal ultrasonography 30 days after insemination. For the Statistical analysis of the variables, we applied the Chi-square test, using the 5% significance level (SPSS - version 19). There was no difference in pregnancy rates between MGA and NoMGA groups [52.6% (81/154) vs. 48.1% (62/129); P=0.414]. When analyzed separately, according to the reproductive status prior to the protocol, there was no difference between groups: cyclic: MGA and NoMGA [58.7% (27/46) vs. 52.3% (23/44); P=0.540] and anovular: MGA and NoMGA [50.9% (55/108) vs. 40.0% (34/85); P=0.131]. However, there was a numerical increase in pregnancy rates of anovular cows supplemented with progestogen, when compared with the control. Thus, supplementation with MGA between the 13th and 18th day after TAI did not interfere on pregnancy rates of anovular or cyclic Nellore cows. However, it is necessary to carry out further studies with a greater number of cows per experimental group.

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A128 FTAI, FTET and AI

Pregnancy rate and litter size of English Bulldog bitches inseminated with fresh semen

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Keywords: canine, reproductive biotechniques, vaginal cytology.

The present study aimed to evaluate the estrous cycle of Bulldog bitches using vaginal cytology, and to inseminate these with fresh semen, leading to the determination of pregnancy rate and the size of the litter. Sixteen (16) English Bulldog breed bitches were used, all with good reproductive history and proved fertility, age ranging from 1.5 to 6 years. Associated with the proestrus detection (external signs: vulva edema and a blood-stained discharge) was performed the vaginal cytology in order to determine the oestrus moment, as well was observed the female behavior of male acceptance. The artificial inseminations (AI) started when the vaginal cytology showed 90% of superficial cells, then repeated every 48h until the vaginal cytology change to metaestrous (increasing the nucleated cell counting) and/or the bitch stop to accept the dog mating. Digital manipulation was used to perform the semen collection, all male breeders underwent a previous seminal examination being selected for use only those ejaculates with more than 70% of total motility, vigor 3 and, at least, $200x10^6$ of spermatozoa. The AI was performed using the intravaginal technique with all the collected ejaculate. The gestation confirmation were made by transabdominal ultrasonography between 25 and 30 days after the last AI, the ultrasonographic evaluation after that was carried out periodically in order to determine the best moment to perform the c-section. The vaginal cytology evaluation, associated with the comportamental changes in the bitch was efficient to detect the estrous onset in 100% of the bitches. Fourteen out of the 16 bitches inseminated got pregnant (87.5%). The litters had a mean number of 5.6 puppies, of those 53.1% (42/79) were male and 46.8% (37/79) were female. In conclusion, AI with fresh semen and whole ejaculate lead to good results in terms of fertility and a large litter size in English Bulldog bitches under these experimental conditions.

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A129 FTAI, FTET and AI

Pregnancy rates in Nelore (*Bos taurus indicus*) heifers treated with PGF2α

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Keywords: artificial insemination, estrus, prostaglandin.

The aim of this study was to evaluate the pregnancy rate of Nelore heifers treated with PGF2α. The experiment was conducted in a farm near Paragominas, Para, in July and September of 2011. We used 694 heifers with average and SEM of 24.1±2.0 months and body condition score (ECC) average 3.5 (range 1 to 5). All heifers underwent transrectal ultrasonography, CHISON D600VET model for ovary evaluation. Heifers that had corpus luteum (n=268) received a dose of 1.5 mL (375 mg) prostaglandin (Ciosin®) on day zero (D0) in the morning and were moved to a pasture for estrus observation in the morning (6 to 7 am) and afternoon (17 to 18h). After nine days (D9) the heifers that did not show estrus behavior (n=110) received a second dose of 1.5 mL (375mg) of prostaglandin (Ciosin®) done intramuscularly (IM). Animals identified in estrus were inseminated on average 12 hours later, being AI done during mornings (n=124) or afternoons (n=144). Inseminations were performed from D2 to D9 and from D10 to D16 with the use of semen from two different Nellore sires and 6 AI technician. Pregnancy diagnosis was carried out 35 days after the last insemination using ultrasonography, Model Ultrasonic Transducer - Mindray / DP-2200vet. The data were analyzed with the statistical Chi-square test with 5% significance level, using SAS (2010). Overall, pregnancy rates according to the number of PGF2α and insemination period was evaluated (morning/afternoon). Pregnancy rate was 51.49% (n=138/268) in 16 AI working days. There was difference ($P<0.05$) in pregnancy rates according to the number of PGF2α applications, where only one application of PGF2α was enough to cause estrus induction in 58.96% (n=158/268) of all animals and a subsequent gestation in 56.96% (n=90/158) of the animals receiving just one PGF2α and 43.64% (n=48/110) for two PGF2α treatments. The pregnancy rate for the period of insemination was 45.16% (n=56/138) on the inseminations carried out in the morning and 56.94% (n=82/138) during the afternoon, being observed statistical difference between periods ($P<0.05$). The results show a satisfactory pregnancy rate following conventional artificial insemination program in Nelore heifers treated only with PGF2α. In conclusion, a single application of PGF2α is effective to obtain good pregnancy rates, with afternoon breedings having greater pregnancy results.

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A130 FTAI, FTET and AI

Tolerana® improves the conception rate of inseminated cows

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Keywords: fertility, materno-fetal tolerance, TAI.

Fertility or reproductive efficiency is the most important component of production efficiency on bovine production systems. The economic value of cows reproductive efficacy were 3.24 bigger than other consume characteristics, indicating the importance of reproductive on bovine maternal herds system productivity (MELTON, 1995). Tolerana® is an innovated product that can help on this context, it acts mediating physiologic events of the maternal pregnancy recognition and maternal immune tolerance (communication between embryos and endometrium), that results in reduction of loss pregnancy or improve the embryo mortality prevent in mammals, resulting in great improvement of conception rate in cows. The goal of this project was to compare the conception rate of inseminated cows distributed in 02 groups (Treated and Control), considering that comparison was always done within each herd. In that way, breed, nutritional score and TFAI protocols are not influence factors on comparative conception rate between groups. Beef cows were used in test (n=7000 Nelore breed cows, average score 3 and with 75 days post-partum in average), distributes in 16 different herds and 250 Holstein cows (1 herd, average score 2.5 and 28kg/day of average milk production). The treatment was always randomly distributed and sequential within each pen of synchronized cows (1 was treated, another not, and successively until finish with that group). Statistical analyses were based on T-Test, using a linear model (pregnancy/non pregnancy) within each category (beef/dairy), using SAS System 6.9. Factors like breed, herd and category were not considered in this statistical model. The recommended dose of Tolerana® was 200µg/cow in one administration. The product was filled into 0.25mL French semen straw. The administration of Tolerana® was performed just after AI gun removal, in which another gun was passed through the cervix and the product was deposited on the uterine lumen. Preliminary results showed a gain of 9.4 percent points conception rate of beef cows, and 11.94 percent points in dairy cows. The average conception rate was 46.2% for Control Group and 55.6% for Treated Group. In dairy cows the average conception rate was 22% (Control Group) and 33.94% (treated Group). There are other ongoing experiments, looking for maximal optimization of the product, that is still at experimental stage and registration (MAPA); and thus not commercially available yet. However, with these results it appears that Tolerana® has an enormous potential – with a great impact in bovine reproduction; minimizing costs related to AI implementation and contributing to the genetic progress in bovine herds.

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A131 FTAI, FTET and AI

Intrafollicular transfer of immature oocyte: an option for bovine female multiplication

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Keywords: bovine, embryos, oocyte injection.

The *in vivo* embryo production by superovulation (SOV) and the *in vitro* embryo production (IVP) are the two available options to accelerate female germplasm multiplication. Despite the capacity of SOV to produce embryos of better quality, it must be respected an interval between superovulation protocols, the FSH administration may affect the physiological pattern of follicular dynamics decreasing the embryo production in the subsequent SOV procedures. On the other hand, with the IVP technique, the donor can be submitted to OPU sessions every week. However, oocytes submitted to in vitro production results in poorer quality embryos. The use of a technique that associates the advantages of in vivo and in vitro systems would be the best option for multiplying animals. The technique that fulfills these requirements is the immature oocytes intrafollicular transfer (IOIFT) in which immature oocytes obtained by OPU are injected into a dominant follicle from a synchronized recipient. The aim of this experiment was to evaluate the embryo production using the IOIFT technique. Heifers (n=12) were synchronized with the following protocol: On D-10 a P4 device (Primer®) was inserted together with the administration of 2 ml of estradiol benzoate (RIC-BE®); at D-2 the devices were removed simultaneously to the administration of 2 ml of prostaglandin (Veteglan®); D-1, 1 ml of EB was administered and on D0 estrus was detected. The oocytes were injected into dominant follicles 58 hours after P4 removal. The mean follicular diameter was 14±1.7 mm. The intrafollicular injections were guided by ultrasound equipped with a 7.5MHz vaginal probe (Aloka®) using a modified aspiration system. The system was filled with follicular fluid and attached to a 27G needle. Grade 1 and 2 oocytes were obtained by aspiration of slaughterhouse ovaries. After selection, 25 oocytes were placed in the needle, with 80 µL of follicular fluid. An insulin syringe at the other end of the system served to perform the injections. A single dose of semen was used for artificial insemination (AI), 6 hours after IOIFT and embryos were recovered by uterine flushing 7 days later. Before uterine flushing, CL measurement was performed to confirm if the ovulation had occurred in the ovary where IOIFT was carried out. The CL mean diameter was 20±2.3mm and CL was observed in the same ovary of IOIFT in 9 animals (75%). The average of recovered structures on D7 was 3.7±2.3 per animal, corresponding to 15% of injected oocytes. From the total structures, 58% were viable embryos (Mc, Bi, Bl or Bx), an average of 2.2±1.9 embryos per animal. Despite the low efficiency, IOIFT is a less expensive alternative for cows multiplication, and enables the production of better quality embryos. Moreover, it is an interesting in vivo model for research in the embryology field.

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A132 FTAI, FTET and AI

Melatonin or progesterone treatment in lactating buffaloes during seasonal anestrus

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Keywords: melatonin implant, progesterone, seasonal anestrus.

The effect of treatment with subcutaneous melatonin implants (MEL) or intravaginal progesterone device (P4) in the ovulation induction and pregnancy outcomes in seasonal anestrus buffaloes (spring and summer) subjected to the Ovsynch protocol was evaluated. At the beginning of the experiment (D -20), 139 anestrous buffaloes were divided according to the body condition score, parity, postpartum period, and diameter of the largest ovarian follicle (LF) into one of 3 groups: control (CG, n=46), progesterone (P4G, n=47) and melatonin (MG, n=46). On this day, each MG buffaloes received twelve MEL implants (216mg; Regulin®, Ceva Animal Health, UK). After 20 days (D0), all females received 10µg im (intramuscular) of GnRH (Buserelin acetate, Prorelinn®, Innovare Biotecnologia e Saúde Animal, Brazil), and in the buffaloes from the P4G was inserted an intravaginal P4 device (1g, Cronipres®, Biogenesis-Bagó Animal Health, Brazil), which remained for seven days. In D7, all females received 150µg im of PGF2α (D-Cloprostenol, Croniben®, Biogenesis-Bagó). Two days after (D9), buffaloes received 10µg im of GnRH (Prorelinn®), and 16h later (D10), were submitted to TAI. After ten days (D20), females were subjected to natural mating, to cover the return to estrus. All buffaloes were evaluated by ultrasonography (Mindray DP2200Vet, China) on D -20 to check the presence of corpus luteum (CL), in the D0 and D9 to measure LF diameter, on D7 to assess ovulation rate to 1st GnRH (Ov-rt), on D20 to evaluate the CL percentage and diameter, on D30 for the pregnancy diagnosis and on D60 to quantify pregnancy loss (PgL) and the pregnancy rate for natural mating (PgM). Statistical analysis was performed by GLIMMIX procedure of the SAS®. There were no differences among experimental groups (CG vs. MG vs. P4G) for the analyzed variables, respectively: LF on D0 (11.5±0.3 vs. 12.0±0.3 vs. 11.4±0 3 mm, P=0.37), Ov-rt (45.6 vs. 38.3 vs. 39.1%, P=0.67), LF on D9 (12.9±0.5 vs. 12.9±0.4 vs. 12.8±0.5 mm, P=0.88), PgL (12.9 vs. 0.0 vs. 16.7 %; P=0.40) and PgM (28.6 vs.12.5 vs. 36.6%, P=0.37). The P4G showed higher CL diameter in D20 and pregnancy rate at TAI than the CG and the MG (19.2±0.5 vs.16.6±0.8 vs.16.2±0.6 mm and 66.0 vs. 8.7 vs. 13.0%; P<0.01); higher percentage of CL on D20 and cumulative pregnancy (TAI+PgM) than the CG and similar to the MG (83.0^{a} vs. 54.4^{b} vs. 71.4^{ab} % and 61.7^{a} vs. 34.8^{b} vs. 41.3^{ab} %, P<0.03) and less days open than the CG and the MG (18.5 ± 1.5^{b} vs. 36.1 ± 1.3^{a} vs. 34.2 ± 1.5^{a} days; P<0.01). In conclusion, P4 treatment associated to the Ovsynch protocol promotes better results regarding the association with MEL in terms of the CL diameter and pregnancy rates, reducing the days open in lactating buffaloes during the seasonal anestrus.

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A133 FTAI, FTET and AI

Treatment with prostaglandin at the beginning of the protocol in beef cows with CL increases conception rate after FTAI

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Keywords: corpus luteum, prostaglandin, TAI.

In beef cows the concentrations of progesterone (P4) before the proestrus is associated with the size of preovulatory follicle that is positively associated with fertility. Thus, strategies that manipulated the concentration of P4 during the fixed-time artificial insemination (TAI) protocol could be useful to improve the results of TAI programs at commercial operations. A simple way the make this is to add a dose of prostaglandin (PGF) on the beginning of TAI protocol (P4 device insertion) of cycling cows. Thus, this work aimed to compare the conception rate at 30 days (CR) of beef cattle after TAI with or without corpus luteum (CL) at the beginning of the protocol. On a random day of the estrus cycle (Day 0) a total of 3,134 beef cows (Exp. 1, 10 different farms, 1,001 cows with CL and 2,133 cows without CL) received a P4 intravaginal device (Sincrogest®, Ourofino Saúde Animal) and 2 mg i.m. of estradiol benzoate (Sincrodiol®, Ourofino Saúde Animal). On Day 8, P4 device was removed and cows received 500 µg i.m. of sodium cloprostenol (Sincrocio®, Ourofino Saúde Animal), 1 mg i.m. of estradiol cypionate (SincroCP®, Ourofino Saúde Animal) and 300 IU i.m. of eCG (SincroeCG®, Ourofino Saúde Animal). TAI was performed on Day 10. In Exp. 2, 3,802 cows (14 different farms, 1,374 cows with CL and 2,428 cows without CL) received the same protocol previously described; however, a dose of 500 µg i.m. of cloprostenol sodium (Sincrocio®, Ourofino Saúde Animal) was administrated on Day 0 in cows presenting CL at the beginning of the protocol. The GLIMMIX procedure (SAS) was used for statistical analysis. In Exp.1, no difference (P=0.08) was observed on the CR between cows with (48% [N=1,001]) or without (51.3% [N=2,133]) CL. On the other hand, in Exp.2 (PGF on Day 0 in cows with CL) the group of cows with CL on Day 0 presented higher CR (55.5% [N=1,374] vs. 52.1 [N=2,428], P=0.05). Farm effect was not detected. In conclusion, similar CR was observed between cows with or without CL at the beginning of protocol when PGF was not used at D0. Also, the addition of a dose of PGF at the beginning of the protocol in cows with CL increased the CR in comparison to other experimental group.

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A134 FTAI, FTET and AI

Different antibiotics associated or not to sodium cloprostenol for treatment of puerperal problems in dairy cows

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UNIFENAS.

Keywords: antibiotic treatment, cefquinome, cloprostenol.

Uterine infections are among the main causes of reduced fertility in dairy cows, both in cases of natural mating, conventional insemination, and fixed time (TAI). The objective of this study was to evaluate the efficacy of two antibiotics, associated or not to sodium cloprostenol in the treatment of puerperal infections in dairy cows. The cows were divided evenly according to parity and body condition score and all treatments were applied intramuscularly, as follow: T1 (n=25): cefquinome (Cobactan® - MSD, Brazil), 1ml / 25kg body weight (BW) once a day for 3 days, T2 (n=25): cefquinome 1ml / 25 kg BW – once a day for 3 days plus 0.5 mg of sodium cloprostenol (Ciosin® - MSD, Brazil) on the 1st and 3rd day of treatment, T3 (n=25): oxytetracycline hydrochloride (Terramycin LA®, Zoetis, Brazil) for two days at 48 hours interval, and T4 (n=25) oxytetracycline for two days at 48 hours interval plus 0.5mg cloprostenol on the 1st and 3rd day of treatment. The animals were re-assessed between the 3rd and 5th weeks postpartum by rectal palpation and ultrasound vaginoscopy to assess the presence and degree of uterine infection. We evaluated the conception rate at 1st insemination, the number of pregnant animals up to 250 days after birth, the average number of services/conception, the calving interval at the 1st artificial insemination (AI) and services period. The trial was carried in a factorial arrangement 2x2 and differences between means were declared at 5% of significance. Significant interactions between the factors studied were not observed. The percentage of infection between the 3rd and 5th weeks was 36.0^{a}; 24.0^{a}; 64.0^{b} and 48.0^{ab}. The conception rate at the 1st AI was 24.0; 32.0; 16.0 and 20.0 (P>0.05). The percentage of pregnant dairy cows up to 250 days postpartum was 76.0^{bc}; 88.0^{c}; 56.0^{a} and 68.0^{ab}. The calving interval at the 1st AI was 67.7 ± 18.6^{b}; 53.2 ± 13.8^{a}; 74.0 ± 22.7^{c} and 64.7 ± 15.1^{b} days. The number of services/conception was 3.2±1.8; 2.9±1.5; 3.5±2.0 and 3.3±1.9 (P>0.05), whereas the services period was 158.5 ± 86.4^{b}; 144.6 ± 72.0^{a}; 179.8 ± 96.2^{c} and 161.3 ± 81.2^{b} days for the treatments T1, T2, T3 and T4, respectively. Comparing the same antibiotic, the association with cloprostenol improved the service period in all cases (P>0.05). We concluded that cefquinome is more efficient than oxytetracycline hydrochloride in the treatment of postpartum uterine infections in dairy cows. The association between antibiotics with an analog of prostaglandin can improve reproductive performance of dairy cows.

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A135 FTAI, FTET and AI

Use of Bioabortogen H® and Bioleptogen® as a strategy to reduce gestational loss between 30 and 60 days of beef heifers

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Keywords: abortion, immunization, reproductive efficiency.

The aim of the study was to evaluate the effect of using reproductive vaccines Bioabortogen H® and Bioleptogen® (protection against IBR, BVD and leptospirosis) previously (D-40 and D0) or during and after the TAI protocol (D0 and D40) of beef heifers on 30 and 60d conception rates and pregnancy loss. The study was conducted at Farm Vera Cruz IV, Barra do Garças – MT. Heifers (n=700) aging between 15 and 24mo were evaluated by ultrasonography and if considered prepubertal (immature uterus and absence of CL) they were subjected to a treatment to induce cyclicity 40d before starting the TAI protocol [D-40: insertion of one 0.558g P4 device (Cronipres Mono Dose®, Biogénesis Bagó, Brazil) previously used for 8d, and D-30: device removal and administration of 1 mg estradiol benzoate (EB; Bioestrogen®, Biogenésis Bagó, Brazil)]. On that day, both cyclic and non-cyclic heifers were homogeneously allocated (cyclicity, ECC and live weight) to receive (VacBefore) or not (VacD0 and control) the first dose of both vaccine Bioabortogen H® and Bioleptogen® (5 mL SC each, Biogenésis Bagó, Brazil). VacBefore heifers received the booster of both vaccines on D0, the beginning of TAI protocol. VacD0 heifers received the first dose of both vaccines on D0 and the booster on D40, the day of pregnancy diagnosis 30d after TAI. Control heifers did not receive vaccination. All heifers were reassessed for cyclicity on D0 and only those considered pubertal (n=619) were kept in the study. The TAI protocol consisted in the insertion of an intravaginal device with 0.558g P4 and 2 mg EB on D0; device removal and administration of 0.150 mg of D-Cloprostenol (Croniben®, Biogénesis Bagó, Brazil) and 300 IU of eCG (Novormon®, MSD, Brazil) on D8; administration of 1 mg EB 24h after (D9) and TAI 48h after device removal. Pregnancy diagnosis was performed 30 and 60d after TAI through ultrasonography (Mindray DP2200VET). Data analysis was done by logistic regression (SAS PROC GLIMMIX). Similar average BCS (P=0.97) and body weight (P=0.48) were observed for heifers from control group (3.02±0.02, and 297.6±3.1), VacBefore (3.02±0.01 and 301.4±3.3) and VacD0 (3.02±0.02 and 299.4±4.3), demonstrating the homogeneity between groups. There was no difference in pregnancy rates at 30 [(control: 37.0% (71/192), VacBefore: 40.5% (90/222) and VacD0: 35.1% (72/205); P=0.55] and 60 days post-TAI [(control: 33.3% (64/192), VacBefore: 40.1% (89/222) and VacD0: 34.2% (70/205); P=0.33]. However, pregnancy loss between 30 and 60d of gestation was considerably inferior in heifers from VacBefore [1.1%^{b} (1/90)] compared to control [9.9% (7/71)^{a}] and similar to VacD0 [2.8%^{ab} (2/72);. P=0.05] Therefore, despite the use of Bioabortogen H® and Bioleptogen®, vaccines did not interfere in pregnancy rates 30 and 60d after TAI. The use was effective to reduce pregnancy loss during this period, especially when used previously to the TAI.

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A136 FTAI, FTET and AI

Use of two doses of cloprostenol at different intervals for estrus synchronization in Santa Inês ewes

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Keywords: ovine, ovulation, prostaglandin.

The objective of this study was to compare protocols for estrus synchronization, using two doses of cloprostenol in different intervals, in Martch, during the breeding season, in Santa Inês ewes, in the city of Cachoeiras de Macacu-RJ. A total of 30 ewes (43.9±6.4 kg, 2.9±0.27 BCS and 3.4±1.6 years old) weaned at least for three months was equally allocated into three treatments, with intervals of: 11.5 days (G11.5: n=10), 9 days (G9: n=10) or 7 days (G7: n=10). The dose used for each administration was 37.5 µg cloprostenol (Estron®, Agener União, São Paulo, Brazil) intramuscularly. Transrectal ultrasound evaluations were performed (B-mode; SonoScape®, Shenzhen, China) for monitoring the follicular and luteal dynamic, daily from 5 d before the first dose, and every 12 h after both administrations, again for 5 d or until ovulation. For estrus detection, females were teased individually every 12 h after each cloprostenol administration for 5 d and for 5 min per animal. Normal quantitative variables were subjected to ANOVA followed by Tukey test ($P<0.05$). Data concerning the rate of ewes in estrus (%) were evaluated by Fisher's exact test ($P<0.05$). The percentage of animals in estrus after the first dose did not differ ($P>0.05$) among groups: G11.5 – 60% (6/10); G9 – 80% (8/10) and G7: 80% (8/10) as well as the duration of estrus (G11.5: 24.0±13.1 h; G9: 37.5±7.7h and G7: 28.5±11.0 h. After the second dose, estrus presentation rates and duration of estrus also did not differ ($P>0.05$) among groups, respectively: G11.5 – 90% (9/10) and 29.3±12.2 h; G9 – 100% (10/10) and 36.0±10.4 h; and G7 – 80% (8/10) and 31.5±8.9 h. There was no statistical difference ($P>0.05$) in the intervals from the second dose to the onset of estrus, end of estrus, and from estrus to ovulation between G11.5 (48.5±8.9 h; 80.0±8.5 h; 35.0±20.1 h), G9 (50.3±13.1 h; 83.0±9.1 h; 25.5±12.2 h) and G7 (36.5±6.2 h; 68.0±14.0 h and 20.3±6.1 h). The interval from the administration of the second dose to ovulation differed ($P<0.05$) among groups G11.5 (78.7±9.4 h), G9 (75.5±8.3) h e G7 (56.8±6.2 h). The G7 anticipated ovulation and presented a lower standard deviation ($P<0.05$). In conclusion, it appears that animals in the G7 were close to follicular dominance period or were already with the dominant follicles while G11.5 and G9 might still be in the beginning of the follicular wave. The three protocols were effective for estrus synchronization in Santa Inês ewes.

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A137 FTAI, FTET and AI

Vetscore: a new method for nutritional assessment and selection of beef cows to TAI

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Keywords: body condiction score, cows, TAI.

Reproductive performance of suckled beef cows is directly associated to their nutritional status. Cows with adequate body condition score (BCS) at timed artificial insemination (TAI) are more likely to become pregnant than cows with low BCS. Although the benefits that BCS evaluation may represent in selecting cows for TAI, the method still consists in visual observation and, therefore, subjective. Based on that, the objectives of this study were to: 1) evaluate the relationship between the angle formed between the sides of the rump and BCS; and, 2) develop an objective tool to select cows for TAI according to their nutritional status. In Experiment 1, 801 suckled Nelore cows of 3 to 12 years old and weighing 400 - 625 Kg. All females were evaluated according to their BCS (1 to 5 scale; 1=emaciated e 5=obese; Ferguson et al., J Dairy Sci, v.77, p.2695-2703, 1994). Moreover, the angle formed between the sides of the rump was measured in all cows with a goniometer instrument. All analysis were performed in SAS 9.0 statistical software. The relationship between BCS and the rump angles was analyzed by regression models. There is a positive relationship between BCS and angle of the rump ($P<0.0001$), whereas the linear regression equation was Angle=79.78 ± 9.53 x BCS; R^2=0,54. Based on the results from Experiment 1, a device that aims to evaluate the nutritional status in a simple, direct and objective way was developed for use in the Experiment 2. This device, known as Vetscore (INPI registration n. 1020140049916), evaluates the BCS according to the rump angle and it classifies cows into 3 different nutritional status, such as: Red, cows with BCS <2.75; Green, cows with BCS between 2.75 and 4.25; and Yellow, cows with BCS >4.25. In this study, 354 Nelore multiparous suckled cows, 4 - 8 years old, were subjected to a TAI protocol (2 mg BE + CIDR at D0 / 2 mL PGF + 300 IU eCG + 1 mg ECP – CIDR at D8 / TAI at 48 h). On D0 all cows were evaluated with the Vetscore and were classified according to its nutritional scale. The pregnancy status was detected with ultrasound 30 d after TAI. Pregnancy rate was analyzed using the Chi-square test. Vetscore presented a precision of 80% and cows classified as Green had higher pregnancy rate than cows classified as Red and Yellow ($P<0.01$). The pregnancy rates according to Vetscore were 38.6% (51/132), 56.74% (122/215), and 28.6% (2/7), for Red, Green, and Yellow, respectively. These results demonstrate that the new methodology to evaluate nutritional status and select cows for TAI is proven efficient. Therefore, Vetscore device may easily be applied to livestock's production system.

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A138 OPU-IVP and ET

Long action progesterone application interferes with recovery and quality in oocyte production and embryos *in vitro* prepubertal Nelore heifers

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Keywords: bovine, oocytes, puberty.

Progesterone has been used for various purposes in the animal reproduction. P4 allows the follicle to be exposed for a longer period to short LH pulses, which may improve oocyte quality and thus the production of embryos. The aim of this study was to evaluate the influence of exogenous progesterone injection in the number of recovered oocyte, oocyte quality and embryo production of prepubertal Nellore heifers. We used 31 females aging from 16 to 18 months, non-pregnant and without corpus luteum (CL). Cows were divided into three groups in a cross-over design: Group P0 (n=11), in which animals received two placebo solution applications (1 mL), in a 7-day interval and starting 14 days (d-14) before the first aspiration (d-0); Group P7 (n=10), the treated group in which the animals received a placebo solution injection (1 mL) 14 days (d-14) and a progesterone injection (P4; 150 mg) 7 days (d-7) before aspiration; Group P7-14 (n = 10), in which animals received two P4 injections (150mμg) with an interval of 7 days, the first one 14 days (d-14) and the second one 7 days (d-7) before aspiration. We conducted three aspirations with 28-day intervals. At the first aspiration animals were divided so that all cows could pass through all treatments. The recovered oocytes were selected and submitted to the procedures of the *in vitro* embryo production (IVEP). After confirming the homocedasticity (BoxCox) and normality (Cramér-von Mises test) of the data, the analysis of variance (ANOVA) was carried out. Tukey's test was used to compare the means of the variables and the Pearson's correlation test was used for data correlations, considering significant when $p \leq 0.05$. There were no significant differences ($p > 0.05$) in the number of retrieved oocytes (total oocytes), viable oocytes (GI, II, III), between the animals treated (P7 and P7-14 groups) and animals that did not receive P4 (P0 group). The animals showed an average of 14.98 ± 10.82 oocytes collected by aspiration session. The mean and standard deviation of viable oocytes rates and rates of embryos produced did not differ ($p > 0.05$) between groups. The groups P0, P7, P7-14 had 76%, 80% and 68% of viable oocytes, and 36%, 42% and 43% of embryos produced, respectively. The groups P0, P7 and P7-14 had an average of 4.04, 5.03 and 4.43 embryos produced by aspiration, respectively. The use of progesterone therapy did not improve the oocytes' and embryonic variables analyzed.

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A139 OPU-IVP and ET

Evaluation off *in vitro* embryos production (IVP) of buffaloes using medium supplemented with essential oil lippia origanoides

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Keywords: antioxidant, buffaloes, embyos.

The *in vitro* embryo production (IVEP) in cattle is a widely used and quite successful biotechnology. On the other hand, in buffaloes the IVEP is still limited and provides low production rates. The whole process that has being done in buffalo is very similar to cattle. One hypothesis for the low production in buffaloes is related to the *in vitro* environment, which generally exposes the oocytes to high oxygen tension and visible light causing an increase in reactive oxygen species (ROS). A strategy to minimize these effects is the use of antioxidants in culture media. A powerful natural antioxidant already used in cell culture is extracted from the plant *Lippia origanoides*. Thus, the aim of this study was to evaluate the effect of supplementation on IVM medium with essential oil from *L. origanoides* (OELO) in different concentrations for buffaloes, using the bovine model as standard. For maturation, we used 2052 bovine oocytes and 1026 buffalo oocytes recovered from ovaries originating from slaughterhouse, divided into five treatments consisting of: T1 [Base Media (BM: TCM 199 + 10% FBS + 22µg / ml pyruvate + 5UI / mL LH + 0,05µg / mL FSH + 1µg / mL Estradiol + 83.4 mg / mL amikacin)], T2 (BM 50 uM / ml cysteamine), T3 (BM + 2,5µg / ml OELO), T4 (BM + 5 ug / ml OELO) and T5 (BM + 10 ug / ml OELO). The reagents used were purchased from Sigma-Aldrich®, St. Louis, USA. Oocytes were maturated at 5% CO2, 38.5 °C for 24 hours. IVF occurred in a period of 18-20 hours, using semen from the same batch. The zygotes were denuded and cultured in SOF medium + 2.5% FBS for 7 days. The cleavage rate was evaluated after 48 hours of culture and the rate of blastocysts production in days 7 and 8. For statistical analysis, the Shapiro-Wilk test was used to assess the normality of continuous variables. The mean comparison between buffaloes and the standard (cattle) was made with ANOVA and Tukey test. The level of significance was $p < 0.05$. The cleavage rates (mean ± standard deviation; %) were 39.9 ± 5.5; 35.6 ± 5.6; 44.1 ± 5.7; 45.2 ± 5.6 and 43.6 ± 6.9 for buffaloes and 64.0 ± 3.9; 62.6 ± 3.6; 64.7 ± 4.0; 53.3 ± 3.8 and 65.5 ± 4.9 for cattle. The blastocyst rates were 30.2 ± 6.5; 19.5 ± 5.9; 27.5 ± 5.1; 32.9 ± 6.5 and 23.3 ± 4.8 for buffaloes and 27.2 ± 2.9; 26.8 ± 3.9; 24.9 ± 3.5; 23.4 ± 2.6 and 27.1 ± 2.9 for cattle, for the treatments T1, T2, T3, T4 and T5, respectively. There was significant difference only in cleavage rates for treatments T1 and T2, and cattle had a best performance compared to buffaloes. The semen used was from a bull with proven fertility in IVEP, not influencing the difference in the production rates between the two species. However, when using the antioxidant essential oil from *L. origanoides*, regardless the concentration, the difference between the two species was eliminated. The use of essential oil of *L. origanoides* was effective to optimize the *in vitro* embryo production in buffaloes. Financial support - CAPES 096/10.

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A140 OPU-IVP and ET

Evaluation of *in vitro* embryo production according to the cyclicity of Nellore (*Bos indicus*) cows submitted to opu in different postpartum moments

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Keywords: corpus luteum, ovum pick up, postpartum.

The aim of the present study was to evaluate the effect of postpartum days and cyclicity at 45 days postpartum on *in vitro* embryo production. A total of 17 Nellore cows, presenting eutocic labor and physiological postpartum, were used and were maintained with calves along all experimental period. Females were submitted to ovum pickup (OPU; 20G; 90 mmHg; ALOKA SSDV 500), in the following postpartum moments: T1- 11.5 days; T2- 25.5 days; T3- 39.5 days; T4- 53.5 days; T5- 66,8 days and T6- 81.4 days. The means (±SEM) of body condition score (1-5) during the postpartum moments were: T1: 2.72±0.04; T2: 2.82±0.04; T3: 2.82±0.04; T4: 2.83±0.05; T5: 2.84±0.04 and T6: 2.88±0.06. Immediately before the OPU, all visible follicles (≥2mm) were quantified and classified according to the diameter [small follicles (SF = <6mm), medium follicles (MF = 6 to 10 mm) and large follicles (LF ≥ 10mm)]. The variables were analyzed using PROC GLIMMIX (SAS 9.2®). Cyclicity effects [presence (C) or absence (NC) of corpus luteum] at 45 days, days postpartum and the interaction of these factors were evaluated. There was no interaction in any variables analyzed. Ciclicity did not influence the number of aspirated follicles (NC:38.3±3.2; C:44.5±2.9; P=0.35), cleavage rate (NC=56.7% e C=60.0%; P=0.30) and blastocyst rate (NC=30.2% e C=34.3%; P=0.37). Concerning postpartum period, there was a higher number of aspirated follicles when the animals were aspirated 81.4 days postpartum compared to previous moments (T1:36.8±4,8c; T2:35.8±4,9c; T3:37.8±4, 4cb; T4:38.6±4,7cb; T5: 43.4±5,6b; T6: 54.2±6,9a; P<0.001). There were also differences between the periods in oocyte recovery rate (T1:88.6%a; T2:73.3%b; T3:73.3%b; T4:81.4%ab; T5:87.1%a; T6:79.4%ab; P=0.03). Regarding the blastocyst rate, animals aspirated 11.5 days after calving showed lower values compared to other periods (T1:23.3%b; T2:34.9%a; T3:38.9%a; T4:33.7%a; T5:32.5%a; T6:31.6%a, P=0.01). However, the number of blastocysts (T1:7.6±1.5; T2:9.2±2.2 ; T3:10.8±2.2; T4:10.6±2.1; T5:12.3±2.2; T6:13.6±2.7; P=0.06), cleavage rate (T1:60.4%; T2:49.0%; T3:61.7%; T4:64.7%; T5:59.8%; T6:54.1%; P=0.08) and the number of cleaved embryos (T1:19.7±3.2; T2:12.9±2.8; T3:17.1±3.0; T4:20.4±3.3; T5:22.6±4.2; T6:23.3±4.6; P=0.10) per OPU session did not differ between different postpartum periods. Thus, although the effect of cyclicity at 45 days postpartum had no effect on *in vitro* production of Nellore embryos, the postpartum period significantly affected the number of follicles aspirated, the oocyte recovery rate and the blastocyst rate.

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A141 OPU-IVP and ET

Evaluation of *in vitro* embryo production in Nelore (*Bos indicus*) with high and low follicular counting submitted to OPU in different postpartum moments

C.R.A. Silveira[1], J.C. Pierucci[1], G.L. Santos[2], W. Yamazaki[2], L.T.S. Yamazaki[2], E.A.R. Dias[3], L.M. Vieira[4], F.M. Monteiro[3], P.S. Baruselli[4], L.U. Gimenes[1]

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Keywords: beef cows, *In vitro* embryo, postpartum.

The present study aimed to evaluate the effect of follicular population, according to the postpartum period, on *in vitro* embryo production in Nellore donors. A total of 17 Nellore cows, presenting eutocic labor and physiological postpartum, were used and were maintained with calves along all experimental period. Females were submitted to ovum pickup (OPU; 20G; 90 mmHg; Aloka SSDV 500), in the following postpartum moments: T1- 11.5 days; T2- 25.5 days; T3- 39.5 days; T4- 53.5 days; T5- 66,8 days and T6- 81.4 days. The means (±SEM) of body condition score (1-5) during the postpartum moments were: T1: 2.72±0.04; T2: 2.82±0.04; T3: 2.82±0.04; T4: 2.83±0.05; T5: 2.84±0.04 and T6: 2.88±0.06. Immediately before the OPU, all visible follicles (≥2mm) were quantified and the animals were separated according to the counting of follicular population, into: High (HFP, 52.9 ± 5.5 antral follicles; n=9) and Low (LFP; 20.8 ± 2.5 antral follicles; n=8) groups. Variables were analyzed using the GLIMMIX procedure (SAS 9.3®). The percentages were calculated based on total number of oocytes. Animals with high follicular population had a greater number of total oocytes (HFP:44.7±3.1; LFP:20.9±1.9; P=0.004), and number of cleaved embryos (HFP:25.4±2.3; LFP:12.9±1.3; P=0.05) compared to low follicular count animals. Interaction between the effects of follicular population count and the period in which the cows were aspirated regarding the cleavage rate (HFP/T1:58.0%abc; HFP/T2:47.7%c; HFP/T3:61.8%ab; HFP/T4:65.0%ab; HFP/T5:62.9%ab; HFP/T6:47.2%c; LFP/T1:66.9%ab; LFP/T2:51.3%bc; LFP/T3:61.8%abc; LFP/T4:64.8%ab; LFP/T5:53.1%bc; LFP/T6:70.3%a; P=0.05) was observed, as well as number of blastocysts (HFP/T1:10.2±2.6ab; HFP/T2:12.9±3.9ab; HFP/T3:15.0±3.3a; HFP/T4:14.4±3.7a;HFP/T5:13.8±2.8a; HFP/T6:14.1±4.2ab; LFP/T1:5.0±1.0b; LFP/T2:5.4±1.6b; LFP/T3:6.7±2.5b; LFP/T4:6.8±1.2ab; LFP/T5:10.6±3.4ab; LFP/T6:13.0±3.7a; P=0.01) and blastocyst rate (HFP/T1:21.3%d; HFP/T2:34.8%abcd; HFP/T3:40.3%ab; HFP/T4:35.7%abc; HFP/T5:28.6%bcd; HFP/T6:24.6%cd; LFP/T1:29.1%bcd; LFP/T2:34.6%abcd; LFP/T3:37.0%abc; LFP/T4:30.0%bcd; LFP/T5:40.8%ab; LFP/T6:47.6%a; P=0.001). Regarding the time of postpartum in which cows were aspirated, regardless of their follicle population, there was difference on the number of total occytes according to the postpartum periods (T1:32.6±5.4bc; T2:26.3±4.6d; T3:27.7±4.5cd; T4:31.5±4.7bc; T5:37.8±6.1ab; T6:43.1±6.1a; P<0.0001) and oocyte recovery rate (T1:88.5%a; T2:73.3%b; T3:73.3%b; T4:81.4%ab; T5:87.1%a; T6:79.4%ab; P=0.03). Thus, it is concluded that the quantity of recovered oocytes was higher in animals with higher follicular count. Additionally, in cows with high follicular count, blastocyst rate was higher in the period between 25.5 and 53.5 days postpartum, while in low follicular count animals, the rate was higher from 25.5 to 39.5 days and between 66.8 and 81.4 days postpartum.

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A142 OPU-IVP and ET

Use of microwave as an alternative method for sterilization of materials used in assisted animal reproduction

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Keywords: *Escherichia coli*, microbiology, *Staphylococcus aureus.*

The plastic material is routinely used in animal assisted reproduction, being disposable which increases the cost of embryos production. Ethylene oxide sterilization is effective, but the process is time consuming, expensive and has high carcinogenic potential. Thus, the use of microwaves as an alternative, is commonly applied by working professionals in this area. However, few reports relate sterilization efficacy. The study aimed to evaluate the microwave efficiency as a sterilization method. The methodology consisted of manual washing of plastic material (with detergent), oven drying, packaging and use of micro-waves (2450MHz) for 5 minutes, twice. A container with 500 mL of water was used, and the water was replaced by another at room temperature between stages. Two assays have been proposed; the first evaluated the efficacy of the process of eliminating two types of micro-organisms whilst the efficiency; in the second, filter embryo collectors sterilization. Two cryovials were contaminated with 10^8 UFC/mL of Staphylococcus aureus and two with the same charge of Escherichia coli, and were randomly divided into 4 groups, two of which were subjected to direct cultivation in Muller Hinton (MH) medium and the other two passed through the microwave before cultivation. The second stage consisted of bovine uterine fluid transcervical collection, female bovine was previously anesthetized. The urethral probe was positioned in the uterus body and a system coupled to "Y" with saline solution at one end and at the other an Erlenmeyer, whereof two liters of solution were drained. The content was homogenized and distributed random and equally in 12 embryo collectors filters. The liquid passed through the mesh and was despised. Four groups were outlined with 3 filters each. Group (G) 1: The filter did not suffered any type of cleaning and followed for cultivation; G2: Passed through microwave and further cultivation; G3: manual washing, drying and cultivation; G4: manual washing, drying, microwave and cultivation. The materials (cryotubes and filters) remained in MH culture for 48 hours at 37 °C. The evaluation criteria were turbidity of the medium after 24 and 48 hours of incubation compared to the negative control and spreading all on 5% sheep blood agar. In the first experiment, there was turbidity in 24 hours and bacterial growth of the cryovials was compatible with the inoculated bacteria. In the second experiment, all groups showed turbidity in 24 hours. Given the results, the use of microwave in the stipulated frequency and time, was not effective in sterilizing the selected materials

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A143 OPU-IVP and ET

In vitro sperm capacitation with l-Arginine and heparin in the absence of cumulus-oocyte complexes and its impact on embryo production

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Keywords: l-arginine, embryos, sperm capacitation.

The aim of this study was to evaluate the effects of L-arginine (L-arg, a precursor of NO synthesis) in the quality of the *in vitro* capacitation of cryopreserved bovine sperm induced by heparin and its effects on *in vitro* embryo production. All reagents used in the capacitation and sperm tests were purchased from Sigma Chemical Co. Aldrick (St Louis, USA). The medium base used in the sperm tests was modified Tyrodes (TALP-sp). The wash medium used in sperm capacitation was Tyrodes (Chamberland, Theriogenology, vol. 55, p. 823-835, 2001). The medium used in the process of *in vitro* embryo production were obtained from Progest Biotechnology in Animal Reproduction Co. (Botucatu, Brazil). In experiment 1, the experimental groups were: control 0h without pre capacitation, and capacitated for 30 min in the absence of COCs with heparin (control 30 min); L-arg and L-arg + heparin. The sperm capacitation and acrosome reaction (AR) were evaluated by chlortetracycline test, and the integrity of the plasma membrane (PM) and acrosome (AM) by the association of three fluorescent probes (PI; Hoechst, FITC-PSA). The sperm capacitation with L-arg + heparin increased the percentage of capacitated sperm, compared to control 0h and the group capacitated with heparin (61,1 vs 18.24 e 47.01%, respectively), and decreased the AR (19.6 vs 25.20%) compared to the group capacitated with heparin ($P < 0.05$). There was no difference in the percentage of sperm with PM and AM integrity when compared with the control 0h ($P > 0.05$). In experiment 2, the sperm was incubated with COCs in the presence of heparin (control), or previously incubated in the absence of COCs for 30 min with heparin, L-Arg or L-Arg + heparin and then washed and transferred to the IVF droplets without heparin. The sperm quality was evaluated by the *in vitro* production rate of blastocysts. There was no significant difference in cleavage rate between treatments ($P > 0.05$), however the group capacitated with L-arg + heparin increased the blastocyst rate at 31.8% compared to the control group, capacitated with heparin in the presence of COCs (53.71 vs 40.76%, $P<0.05$). The results allow us to conclude that: 1) the addition of L-arg to the capacitation medium containing heparin increases the number of *in vitro* capacitated spermatozoa with 30 min of cultive; 2) maintains a low percentage of spermatozoa with damaged in the plasma membrane and acrosomal membrane and 3) the addition of L-arg to the capacitation medium with heparin, in the absence of COCs, was the most effective method in the blastocysts production.

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A144 OPU-IVP and ET

Comparison of ovarian storage different solutions on qualitative-quantitative parameters evaluated by morphological criteria and brilliant cresyl test in bovine oocytes

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Keywords: bcb test, *In vitro* production, oocyte quality.

The objective of this study was to evaluate different solutions [Dulbecco Modified Eagle Medium (DMEM) *vs.* saline (NaCl) *vs.* phosphate buffered saline (PBS)] together with 10% fetal bovine serum (FBS) to refrigerated storage (5°C) of bovine ovaries during 24 h. Thus, ovaries derived from slaughterhouse were transported at 37°C for 15 min and in the laboratory divided in four groups: fresh (control) and cool (5°C) in DMEM/FBS, NaCl/FBS and PBS/FBS solutions. Immediately after ovary recovery (control) or storage (remaining treatments), were aspirated with a needle and syringe (21G/5 mL) follicles (2-8 mm). Then, recovered oocytes were classified in accordance with the *cumulus* cells (CCs) and homogeneity of the cytoplasm Grade I (≥3 layers of CCs and homogeneous cytoplasm), grade II (1-2 layers of CCs and homogeneous cytoplasm), Grade III (<1 layer of CCs and heterogeneous cytoplasm) and Grade IV (degenerated oocyte). Oocytes grade I and II were considered viable while grade III and IV non-viable. After conventional morphological classification, oocytes were submitted to the brilliant cresyl blue stain (Sigma, USA, BCB, 26 μM, 60 min) and classified in BCB^+ (cytoplasm blue/viable oocyte) and BCB^- (colorless cytoplasm/non-viable oocyte). All of the data were analyzed by the Fisher exact test ($P<0.05$). After five repetitions, a total of 120 ovaries resulted in 1047 recovered structures, obtaining an average of 8.8 oocytes/ovary and overall recovery rate (retrieved oocytes / aspirated follicles) of 51.9% (1047/2016). No difference ($P=0.12$) was observed in oocyte recovery rate between the DMEM/FBS [221/463 (47.7%)] and NaCl/FBS groups [157/372 (42.2%)], which were different and the smaller ($P<0.01$) that the control [322/559 (57.6%)] and PBS/FBS groups [347/622 (55.8%)]. As the oocyte quality by morphological criteria, a higher percentage of viable oocytes ($P<0.05$) was obtained from ovaries stored in DMEM/FBS [152/221 (68.8%)] and NaCl/SFB [111/157 (70.7%)] compared to the control [194/322 (60.2%)] and PBS/FBS groups [208/347 (59.9%)], which did not differentiate between them ($P=0.94$). As the oocyte quality by BCB, a percentage similar to control group [131/322 (40.7%)] of viable oocytes was obtained only from ovaries stored in NaCl/FBS [52/157 (33.1%), $P=0.13$]. Differences were observed among the groups; however, while PBS/FBS showed a greater number of recovery oocytes NaCl/FBS showed a higher number of viable oocytes for both oocyte quality assessments. Possibly, simple more solutions for the storage of cold ovaries for 24 h do not affect the quality of recovered structures. In conclusion, the use of NaCl/FBS may be used for storing ovaries at 5°C for 24 h. Further studies are needed to investigate the development of competence of these bovine oocytes.

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A145 OPU-IVP and ET

Comparison of IVF embryo production and pregnancy, using conventional sperm and sex-sorted sperm in cattle

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Keywords: IVF, pregnancy, sex-sorted sperm.

The aim of this study was to evaluate the efficiency of sex-sorted sperm from 4 Nelore bulls on *in vitro* embryo production. For comparison, bovine embryos were also produced by IVF using unsorted sperm from the same bulls. Ovum pick up (OPU) -derived oocytes were matured and fertilized *in vitro* with either unsorted sperm (US) and sex-sorted sperm (SS). Subsequently, the presumptive zygotes were cultured *in vitro* for 7 days under standard conditions, to assess embryonic development rates. Development rates were documented on D7 and pregnancy rates were 35 days after embryo transfer. Our result demonstrated that embryo (Stage 7) production rate in SS Group was lower than US Group (29.0% -2709 blastocysts- vs 37.1% -3,167 blastocysts- respectively. P<0.001). However, the pregnancy rate in SS Group was higher than in the US Group (36.9% vs 30.1%, respectively. P<0.001). Comparing individual performance, from 4 bulls, just one showed higher embryo production rate when was used its sex-sorted semen than when was used its conventional semen (bull I, 26.6% vs 33.5%; bull ll, 40.0% vs 36.3%; bull III 22.3% vs 39.4%; and bull IV, 20.7% vs 35.9%, respectively). However, despite the lower embryo production rate with sex-sorted semen, the pregnancy rates were higher for 3 bulls in the Group SS comparing with Group US (bull I, 42.5% vs 24.1%; bull II 39.7% vs 34.8%; bull III 24.3% vs 36.0%; and bull IV 40.5% vs 33.3%, respectively). In conclusion, based on the results study, despite the embryo production rate was higher for unsorted sperm, the pregnacy rates was superior for sex-sorted sperm group.

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A146 OPU-IVP and ET

Comparison between the number of recovered structures and viable embryos of ewes submitted to superovulation protocol supplemented with glycerol

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Keywords: embryo, energy flushing, reproduction.

The objective was to evaluate the influence of ultrashort energy flushing with glycerol on the amount of recovered structures and viable embryos from superovulated ewes. Sixty of Dorper ewes were submitted to a superovulation protocol that consisted of inserting an intravaginal device containing 0.33 g progesterone (CIDR - G®, Zoetis, São Paulo, Brazil) on Day 0, seven days after the device was replaced and 125µg administered IM sodium cloprostenol. (Ciosin®, MSD Animal Health, São Paulo, Brazil). Ovarian stimulation was started on D12 using 200mg of FSHp in 8 applications spaced 12h in decreasing doses IM (20, 20, 15, 15, 10, 10, 5, 5%). At 6 pm of D14, concomitant 6th application FSHp, IM eCG was administered 200IU (Novormon®, MSD Animal Health, São Paulo, Brazil). At 8 pm of the same day the CIDR - G was removed and the animals were divided into two groups, G-SUP (n = 24) consisting of ewes treated with 100ml glycerol, orally as glycogen energy source for flushing effect of ultrashort and G-CONTROL (n = 36) animals which received the compound for placebo effect 100ml water, orally. At 8 pm the D15 for induction of ovulation was applied 0.1mg IM gonadorelina (Fertagyl®, MSD Animal Health, São Paulo, Brazil). The ewes were inseminated two times (36 and 44h) after removal of the device containing P4, laparoscopic AI was performed using fresh semen and embryos were collected by surgical method on the morning of D21. Statistical analysis was performed using the Stastical Package for Social Sciences (SPSS) version 19, the variables were compared using the *Student's t test*, considering the significance level of 5%. The total average structures and viable embryos in the present study were 5.87 ± 5.97 and 3.73 ± 4.27 respectively. The average yield in total structures and viable embryos G-SUP were 5.83 ± 6.64 and 3.67 ± 4.08 respectively, statistically similar to that obtained in G-CONTROL which was 5.89 ± 5.58 and 3.78 ± 4.45 ($p>0.05$), respectively. In conclusion that supplementation with glycerol in the form of ultrashort energy flushing did not influence the amount of recovered structures and viable embryos in superovulated ewes.

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A147 OPU-IVP and ET

Description of two new surgical techniques for the treatment of vaginal prolapse in zebuine cows: partial vaginectomy and dorsal vaginopexy

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Transfix Transplante de Embriões Ltda.

Keywords: prolapse; vaginectomy; vaginopexy.

Currently, the reduction of vaginal prolapse in non-pregnant zebu cows donors showed high incidence. Conventional techniques such as Caslick, Bühner or Flessa are not efficient and have significant recurrence of this affection. Thus this work proposes the evaluation of two new surgical techniques for correction of vaginal prolapse in female donors, in which terms: partial vaginectomy and dorsal vaginopexy. These techniques were applied in practice for a period of four years, providing up of adult zebu cows (n = 812) of the breeds: Nelore, Gir and Brahman, belonging to different properties. The vaginal prolapse was diagnosed by semiologic evaluation, and further determined the technique to be applied according to the severity and duration of the process. The partial vaginectomy and dorsal vaginopexy procedures demonstrated high percentage of recovery, 93.4% and 96.1%, and low rate of recurrence (6.3% and 3.7%) and low mortality (0.2% and 0.3%), respectively. Therefore, we suggest that the two technical proposals promote permanent reduction of vaginal prolapse in non-pregnant zebu donor females.

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A148 OPU-IVP and ET

Development of *in vitro* produced bovine embryos after cooling in medium 199 and Botuembryo®

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Keywords: cooling, culture media, embryo.

The *in vitro* production of bovine embryos obtained increased annually, but still is faced with a barrier, the lack of synchronized recipients for the time of embryo transfer (ET). Thus, freezing or vitrification of surplus embryos is an option. However, the pregnancy results to date are inconsistent. Recent research suggests the use of refrigerated embryos as a viable alternative and affordable economically. This study proposes the assessment of the effects for the environment and chilling time on the subsequent development of bovine embryos produced *in vitro*. The ovaries used for obtaining oocytes were originating from slaughterhouses. The oocytes were selected and rinsed in PBS with 10% FBS and then matured (IVM; groups 20-25/drop) in Medium 199 plus sodium bicarbonate, sodium pyruvate, penicillin, FCS, FSH and LH for 24 hours. The sperm select was performed by discontinuous gradient centrifugation (45 to 90%) of Percoll®; and the TALP medium supplemented with PHE and heparin for use in *in vitro* fertilization (IVF). The gametes were coincubated for 20 hours and the likely zygotes cultured (MIC) in SOF medium, maintained for seven days. The steps of IVM-IVF and IVC is effected in the incubator with 5% CO_2 in air, 38.7ºC and high humidity. After seven days of IVF, blastocysts were evaluated and randomly divided into five groups, namely blastocysts kept in SOF medium under the same cultivation conditions previously mentioned for the CIV for 6 hours (the control group); chilled blastocysts amid BotuEmbryo® (Botupharma, Botucatu, Brazil), 24 (B24 group) and 48 hours (B48 group); blastocysts and chilled in Medium 199 plus sodium bicarbonate, sodium pyruvate, HEPES (25 mM), FCS (50%) and penicillin, 24 (M24) and 48 hours (M48), in a polystyrene box (BotuFlex®; Botupharma) previously equilibrated at 5° C. After the cooling period, the blastocysts of refrigerated groups 24 and 48 h were transferred to SOF medium and incubated in CO_2 incubator for 6 hours for evaluation of subsequent development. ANOVA and Bonferroni t test were used to assess differences between groups, with $P<0.05$ indicating significance. There was no difference between the control group (100% vs 100%), B24 and M24 (85.6% vs. 65.8%) and B48 and M48 (58.4% vs. 43.8%) to rate blastocyst/expanded blastocyst (B/Bx). However, higher percentage ($P<0.05$) B/Bx was observed between the control group compared to B48 and M48 groups, with similar results between the groups, B24 and M24. Therefore, the BotuEmbryo® medium for 24 hours, proved to be efficient for cooling bovine embryos produced *in vitro*.

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A149 OPU-IVP and ET

Different embryo collection methods and superovulation protocols in crioula lanada ewes

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Keywords: hormone, reproduction, sheep.

The sheep breeds with specific environmental genetic adaptation provides economic benefits for the producer, which requires their preservation. In southern Brazil, the race Creole Lanada presents resistance to endoparasites and foot problems. The inclusion of other races reflected in intersections and spread the breed standard, requiring alternatives to facilitate the maintenance and preservation of genetic adaptations race. This paper proposes to determine the efficiency of different commercial formulations of FSH in superovulation protocol in different ways to collect embryos. Multiparous sheep females (n=8), from the in situ preservation of core Embrapa South Livestock (Bagé, RS), previously selected for cervical catheterization, were subjected to superovulation. It were formed with two groups (n = 4) in which the obtained synchronized estrus cycle, that superovulation occurs 60 hours post-estrus. One group received FSH (200mg, Foltropin V®) and the other FSH + LH (250 + 250, Pluset®) twice daily for four consecutive days. On the first day, it was deployed CIDR-G®; in which it was removed after 72 hours, by the oral application of hyperacute flushing glycerin and PGF2á IM application. The estrus was detected by ruffians and then the females were inseminated (12 and 24), by superficial cervical route with cooled semen (150 x 10^6 cells / mL) two males of the race. Two females from each group were selected for collecting embryos by laparotomy (LT) or via transcervical (TC). On the fifth day after estrus, females were submitted to water and fasting. The flock of the TC collection received an IM dose of BE and oxytocin, 12h and 15min before the procedure, respectively. In D6, the sheep of the LT group were anesthetized (ketamine + xylazine) and submitted to the collection and counting of corpora lutea (CLs). In the TC group, this count was later done by laparoscopy. Females group had FSH response averaged 5.5 LCs, in which the TC group of each animal recovery rate was 50% and 100%, and the rates LT group were 67% and 100%. In females FSH + LH group, the average was 11.2 LC, with recovery rate in the CT group 100% and 0%, and the LT group of 73% and 66%. Most of the collected structures were not fertilized. The collection by LT provided more consistent results, however, via TC provided an acceptable rate of embryo recovery. Because of the negative effects of LT, this result should be considered in races with limited number of copies. The number of LCs obtained with treatment FSH + LH was higher than that obtained with FSH, proposing new reproductive investigations of female sheep of the Creole race.

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A150 OPU-IVP and ET

Cervical dilatation in Santa Ines ewes induced with misoprostol, oxytocin and estradiol for development of non-surgical method for embryo recovery

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Keywords: embryo transfer, sheep, transcervical.

The objective of this study to develop a pharmacological protocol cervical dilatation in ewes Santa Ines aimed at collecting embryos transcervical. Multiparous ewes were used (n=30), cyclic, aged 24 and 48 months, with an average weight of 50.7 ± 5.4 kg and body scores between 2.75 and 3.5 (1-5) in design experimental model in "cross-over". The oestrus of the females was synchronized with short protocol and collection procedures were performed on the 14th day after estrus. Each animal received a dose of 0.5 ml of D-cloprostenol 12 hours before the procedure. All groups received epidural injections of 2.0 mg / kg ketamine (ketamine Agener® Agener Animal Health Union - São Paulo, Brazil) administered in the intervertebral space L7-S1. The experimental groups were: CG = control; GMI = misoprostol; GMiOE = misoprostol, estradiol and oxytocin; GOE = estradiol and oxytocin. The groups who used misoprostol received 5h before the procedure, 200µg of the drug (Prostokos®, Hebron Laboratory, Caruaru, Brazil) diluted in 1.5 mL of saline deposited directly on the cervical ostium. The estradiol benzoate (RIC-BE® Tecnopec, São Paulo, Brazil) was administered intravenously at a dose of 100 ug per animal, diluted in saline 2.5 mL + 2.5 mL of ethanol, 12 hours before the procedure. Oxytocin (Oxytocin Strong UCB®, Centrovet, Goiania, Brazil) was administered intravenously at a dose of 100 IU per animal, 15 minutes before the procedure. The animals were sedated with acepromazine association (0.1mg / kg Acepran®, Vetnil, Louveira, Brazil) and diazepam (0.2 mg / kg diazepam, Teuto Anapolis, Brazil) via IV and after ten minutes, received the injection epidural. The cervix was pinched, pulled to the vulvar commissure and fixed with two clamps to the side Pozzi cervical ostium. Cervical transposition attempts were made with a Hegar candle 10, 20 and 40 minutes after the epidural each trial persisted five minutes. The transposition was confirmed by injecting and recovering from 20 to 40 ml of saline. Data were analyzed in SAEG program (Statistical Analysis System, Version 9.1: Arthur Bernardes Foundation - UFV - Viçosa, 2007) and submitted to Fisher's exact test with 95% significance level. The cervical transposition rate varied between the groups, in which GOE introduced rate of 90% (27/30) did not differ from GMiOE, with a rate of 86.2% (25/30), but demonstrated superiority to other groups. In GMI group was possible to transpose 68.9% (20/29) of cervices equivalent value (P> 0.05) GMiOE and GC (62.1% - 18/29). The study proved the possibility of increasing the cervical transposition rate in ewes Santa Ines with the use of hormonal associations, enabling the collection of embryos transcervical in sheep, avoiding surgery.

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A151 OPU-IVP and ET

Decreased lipid granules of *In vitro* produced bovine embryos with low concentrations of forskolin

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Keywords: Forskolin, IVP, lipids.

This paper proposes the chemical induction of lipolysis addition of forskolin on the sixth day of embryo culture, in order to increase the rate of survival of IVP embryos. Eight replicates were performed using 1.519 oocytes, which were washed and transferred to drops of IVM in TCM 199 supplemented with 10% FCS and remained in an oven with 5% CO_2 in air, at a temperature of 38.5 ° C and humidity absolute for 24 h. Semen was selected by Percoll gradient obtaining a concentration adjusted to $2x10^6$ spermatozoa / mL. Oocytes were incubated for 24 h in incubator with 5% CO^2 in air, at a temperature of 38.5 ° C and absolute humidity. Presumptive zygotes were cultured in SOF and 2.5% FBS. The embryos were kept in an oven with 5% CO_2, 5% O_2 and 90% N_2 at a temperature of 38.5 ° C and absolute humidity D to 6 (0 = D FIV), which were divided time into four groups for the addition of Forskolin® (F-6886): Control (group cultured in the absence of forskolin for seven consecutive days); F 2,5µM (group cultivated with 2,5µM forskolin for 24 hours); F 5µM (group cultivated with 5µM forskolin for 24 hours); F 10µM (group cultivated with 10µM forskolin for 24 hours). Apoptosis was analyzed using the TUNEL technique (deoxinucleotil terminal transferase Uracil Nick End Labeling), and the lipid content analysis was performed with Sudan Black B (S-0395). Statistical analysis Data were analyzed using ANOVA using SAS PROC GLM (SAS Inst., Inc., Cary, NC, USA). Sources of variation in the model including treatment and replicas were regarded as fixed and random effects, respectively. Data are presented as mean and standard least squares error. For all analyzes was adopted the significance level of 5%. There was no difference in blastocyst formation rate: control (37.0 ± 4.0); 2,5µM F (38.6 ± 4.0); F 5µM (40.7 ± 4.0); F 10µM (31.4 ± 4.0). While all groups treated with forskolin showed differences on lipid measurement: control (50.6 ± $1,1^{ab}$); 2,5µM F (46.2 ± $1,1^{c}$); F 5µM (49.9 ± $1,2^{b}$); 10µM F (53.9 ± $1,2^{a}$); total number of whole cells: control (140.1 ± $10,7^{ac}$); F 2,5µM (173 ± $9,0^{b}$); F 5µM (120.6 ± $11,5^{c}$); F 10µM (157.0 ± $13,9^{ab}$) and cell apoptosis: control (12.1 ± 3.5^{a}); 2,5µM F (16.7 ± $4,1^{b}$); F 5µM (11.1 ± $6,5^{a}$); 10µM F (14.2 ± $4,6^{ab}$) ($P < 0.05$). It was concluded that forskolin is a lipolytic agent effective even at low concentrations (F 2.5 mM), leading to the formation of blastocysts with more cells, comparing it to the control group. However, the concentration of 5 uM forskolin induced programmed cell death at the lowest rate.

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A152 OPU-IVP and ET

Effect of addition of butaphosphan to the medium of oocyte maturation in the production of *in vitro* bovine embryos

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Keywords: butafosfan, IVM, IVP.

The quality of the oocyte maturation is directly related to the embryonic development rate, as it depends on the amount of oocyte mRNA stored during this period. During maturation, the oocyte undergoes different processes that are related to phosphorylation and dephosphorylation cascades. Butaphosphan, an organic phosphorus molecule, has been administrated in embryo or oocyte donor cows to improve the results of the assisted reproductive technology programs. Therefore, our hypothesis in this study was the one that the addition of butaphosphan to the *in vitro* maturation medium (IVM) could improve oocyte maturation, enlarging the production of embryos. Eight routines of IVF were performed, totalizing 800 cumulus/oocyte complex originated from slaughterhouses. Mediums of Cenatte® (Cenatte®, Pedro Leopoldo – MG – Brasil) were used to perform IVF. The oocytes were randomly divided, in each routine, into four groups of 25 oocytes and the IVM medium was supplemented with crescent doses of butaphosphan (Capot Chemical Company Limited, Hangzhou, Zhejang, China) (Gc = 0.0mg/L; G1 = 50mg/L; G2 = 100mg/L e G3 = 200mg/L). IVM took place for 24h in an incubator with 5% CO2 and temperature of 39°C. Spermatozoids were selected by the minipercoll method and 10µL with 1x106 spermatozoids/mL were used to perform IVF. After 18h, probable zygotes were transferred to the culture medium under controlled conditions (5% CO_2, 5% O_2, 90% N_2) at 39°C for 7 days. SAS (SAS, Cary, NC, EUA) was used to perform the statistical analysis, GLM was used to verify the linear, squared and cubic effect of the addition of the crescent doses of butaphosphan to the IVM medium over the embryos percentage in D7 in relation to the number of maturated oocytes. The production of embryos in D7 as of 34.7%, 28.5%, 33.5% e 31.6% in the groups Gc, G1, G2 and G3 respectively, there was no effect of the group ($P>0.05$). Our data showed that the addition of butaphosphan to the IVM medium does not interfere directly on oocyte maturation, as it did not affect the production of *in vitro* bovine embryos.

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A153 OPU-IVP and ET

Effect of estrus synchronyzation between embryo donors and recipients, embryo quality and state (fresh or frozen) on the pregnancy rate in enbryo transfer

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Keywords: embryo quality, embryo transfer, estrous synchronization.

The aim of this study was to evaluate the effects of estrous synchrony between donor and the recipient, the quality and the freezability status (fresh or frozen) of the embryo on the pregnancy rate in bovine recipients. The work was performed during two years in an embryo transfer (ET) center, located in the city of Santiago, Rio Grande do Sul, Brazil. Ninety Aberdeen Angus cows were submitted to superovulation (SOV) protocols, resulting in 1.097 ET. The SOV protocol was initiated ten days after the heat detection of donors and the embryos collection was performed, on average, seven days later. The heat of recipients was also observed and on the day of ET, the animals which showed closest synchrony with donors were selected. Thus, 11 groups were formed, with intervals of 6 h, from - 30 to + 30 h, regarding recipient versus donor heat detection. The evaluation of embryos quality followed the proposed by IETS (1998), as grade I- Excellent; II- Regular; III- Poor; IV- Dead or Degenerated. The analysis was performed with the GLM package of the R software, through logistic regression models. The occurrence or not of pregnancy was analyzed as binomial dependent variable. For all the analyses, the significance was considered when $P<0.05$. Also, the odds ratio for each unit increase in the explanatory variables was estimated. The total pregnancy rate was of 52%. There was no effect of synchrony between donor and recipient on pregnancy rate, ranging from 42% to 56% ($P>0.05$). The embryo qualityaffected the pregnancy rate: Grade I, 58%; Grade II, 56%; and, Grade III, 44% of pregnancy ($P<0.001$). As well as the embryo state affected the pregnancy rate, 84% for fresh embryo and 16% for frozen embryo ($P=0.04$). Embryo Grade III had 43% less likelihood of pregnancy than Grade I. Also, frozen embryos had 37% less chance of pregnancy than fresh embryo. The synchrony between donor and recipient, considering ± 30 hours interval did not affect the pregnancy rate. However, the embryo quality and state (fresh or frozen) affected the pregnancy rate.

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A154 OPU-IVP and ET

Effects of rumen-protected methionine and choline supplementation on preimplantation embryo in Holstein cows

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Keywords: choline, embryo, methionine.

Our objective was to determine the effects of methionine and choline supplementation during the pre- and postpartum periods on preimplantation embryos of Holstein cows. Multiparous cows were assigned in a randomized complete block design into four treatments from 21 d before calving to 30 DIM. Treatments (TRT) were: CON (n=8, fed the close-up and fresh cow diets with a Lys:Met=3.5:1), MET (n=9, fed the basal diet + methionine, Smartamine® M to a Lys:Met=2.9:1), CHO (n=8, fed the basal diets + choline 60 g/d, Reashure®), and MIX (n=11, fed the basal diets plus Smartamine M® to a Lys:Met=2.9:1 and 60 g/d Reashure®). From 30 ± 1 to 72 ± 1 DIM the cows were randomly assigned to two groups (GRP); control (CTL; n=16, fed a basal diet with a 3.5:1 Lys:Met) and methionine (SMT; n=20, fed the basal diet + methionine to 2.9:1 Lys:Met). On d 60, dominant follicles greater than 5 mm were aspirated using an ultrasound-guided transvaginal approach. A CIDR® device was inserted in all cows after follicular aspiration and superovulation began at d 61.5 using FSH treatment equivalent to 400 mg of NIHFSH-P1 (Folltropin®) in 8 decreasing doses at 12 h intervals over a 4 d period. During the superovulatory period, all cows received two PGF2α injections at d 63 and d 64 (concomitant with the 5th and 7th FSH injections), and CIDR was withdrawn at d 65. Twenty-four hours after CIDR withdrawal, ovulation was induced with GnRH. Cows were artificially inseminated at 12 h and 24 h after GnRH. Embryos were flushed 6.5 d after artificial insemination. Global methylation of the embryos was assessed by immunofluorescent labeling with 5-methylcytosine, while lipid content was assessed by staining with Nile Red. Nuclear staining (propidium iodide or Hoescht 33342) was used to count the total number of cells/embryo. Statistical analysis was performed using the MIXED procedure of SAS. Methylation of the DNA did was not different (P>0.05) among treatments but there was a TRT × GRP interaction (P=0.03). Embryos from cows in CON (– 21 to 72 d) had greater (P = 0.04) methylation (0.87 ± 0.09) than the embryos from cows in MET and CTL (0.44 ± 0.07).Embryos from cows in SMT had greater lipid content (P=0.04; 7.02 ± 1.03) than CTL (3.61 ± 1.20). There was not difference (P>0.05) for cells/embryo, embryo recovery rate per flushing, number of embryos recovered, embryo quality, embryo stage, and numbers of CL at flushing (CTL: 61.48±5.12, 0.69 %, 7.69±1.39, 1.63±0.25, 3.92±0.12, 9.33±1.18; and SMT: 54.92±3.9, 0.80 %, 8.72±1.18, 1.99±0.20, 4.13±0.10, 11.47±0.99, respectively). In conclusion, supplementation of methionine, choline or both methionine and choline affect embryo methylation and lipid content.

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A155 OPU-IVP and ET

Effect of *in vitro* spom maturation system use on bovine embryos lipid score

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Keywords: bovine embryo, forskolin, Lipidic score.

Oocyte quality determines the proportion of oocytes that will develop to blastocyst stage, and although the lipid content is important in oocyte development, the elevated number of lipid droplets have been associated with reduced cryosurvival, which is a relevant issue for embryo IVP system. The *in vitro* maturation system (IVM) Simulated Physiological Oocyte Maturation (SPOM) mimics the physiological maturation events by using AMPc modulators that promote the increase of oocyte competence. Forskolin is an example of AMPc modulator and this molecule has a lipolytic action. The aim of this study was to evaluate the effect of the SPOM system (Albuz, Hum. Reprod, v25, p12; 2010) on embryonic lipid score (relation between the lipid content and the total number of cells,TNC). In four replicates oocytes were obtained from slaughterhouse ovaries, selected and randomly divided into three groups: SPOM, CONTROL 1 (C1) e CONTROL 2 (C2). The MIV occurred during 24 h in C1 (TCM199 medium without FBS) and C2 (commercial medium Bioklone® Animal Reproduction, Sao Paulo, Brazil/ with FBS) in culture incubator at 38.5° C, 5% CO_2 in atmospheric air and high humidity. In SPOM group, oocytes were incubated in pre-IVM (TCM 199 medium with 100µM Forskolin and 500µM IBMX) for 2 h followed by an extended IVM (TCM 199 medium + 20µM cilostamide) period (28 h) under the same conditions as described for other groups. After IVM, oocytes were fertilized, and transfered to culture droplets, where they remained for seven (n=25-46 per group) or 9 (n=6-9 per group) days. The lipid content analysis and TNC meansure were performed using Oil Red and HOECHST 33342 staining, respectively. The lipid score was obtained by the stained lipid area divided by the TNC of each embryo and the averages were compared according to the days (D7 or D9), for each treatment, by the Kruskal-Wallis test in the Instat GraphPad program, with significance level of 5%. There was no difference ($P<0.05$) between groups (SPOM: 298.5 ± 139.9a ; C1: 226.0 ± 75.7; C2: 211.3 ± 1003a) in D7, suggesting that the time of exposure to Forskolin was not enough to ensure lipolytic action. At D9, only the C2 showed increase compared to others (SPOM: 154.0 ± 27.1a; C1: 135.7 ± 26.2a ; C2: 291.8 ± 71.4b); possibly due to the FBS effect on lipid accumulation. Between D7 and D9, there was a reduction ($P<0.05$) in the lipid score at SPOM (298.5± 139.9 vs 154.0± 27.1) and C1 (226.0 ±75.7 vs 135.7± 26.2) groups, which can be explained by the increase of embryonic metabolic consumption with the advance of embryonic development. However, the C2 showed no difference ($P>0.05$) between day 7 and 9 (211.3 ± 100.3 vs 291.8 ± 71.4), suggesting that the FCS effect on lipid accumulation was greater than the embryos metabolic activity. It was concluded that SPOM system had no effect in lipid score of *in vitro* produced embryos.

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A156 OPU-IVP and ET

The effect of diets with different levels of degradable and undegradable protein in rumen on apoptosis in oocyte and in cumulus-oophurus cells of Girolando cows

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Keywords: nutrition, reproduction, urea.

The ammonia produced during rumen protein degradation and not used in microbial protein synthesis, is absorbed by the rumen wall and converted to urea in the liver. High serum concentrations of ammonia and urea cause increase of both in tissue and reproductive fluids, interfering negatively in the fertility and *in vitro* embryos production. This study evaluated the effect of diets with different levels of degradable protein (RDP) and undegradable protein in rumen (RUP) on apoptosis of oocytes and cumulus-oophorus cells in crossbred Girolando cows. 22 Girolando cows were evaluated (n=10 3/4 HG; n=12 7/8 HG) with average weight 475.8 ± 7.75 kg, BCS 3.22 ± 0.03 and 105.33 ± 23.15 days of postpartum. The animals were distributed in four experimental groups, fed twice daily for 68 days with a total diet based on corn silage and concentrate. The diet of each group varied in the relation between RDP:RUP, with maintenance of metabolizable protein (1888g/day) and reduction of crude protein (CP) (RDP:RUP1.68= 15.4% CP, 62.7% RDP, 37.3% RUP; RDP:RUP 1.31= 13.6% CP, 56.7% RDP, 43.3% RUP; RDP:RUP 1.08= 13% CP, 52% RDP, 48% RUP; RDP:RUP0.83= 12.4% CP, 45.4% RDP, 54.6% RUP). OPU's were done on days 33 and 63 after initiation of treatment. The follicular waves were synchronized at 72 hours before by puncture of all follicles present in the ovaries. The cumulus-oocyte complex (COC) were recovered in PBS medium with 20 UI/mL of heparin, classified as viable (grades 1, 2 and 3) and non-viable. The COC viable were fixed in formalin 10%, alcohol PVA, grouped by treatment, collection day and stained with DAPI and TUNEL (Promega, Wisconsin, USA). The structures were photographed in fluorescence confocal microscope Leica TCS SP5II (Leica Microsystems®, Wetzlar, Germany) at 40x magnification. Images obtained at each 16µm were evaluated in Leica LAS AF Lite software. Getting the number and ratesof the cumulus apoptotic cells and the percentage of apoptotic oocytes. The variables were submitted to analysis of variance, using a generalized linear model (PROC GLM) and means were compared using the Student t test ($P<0.05$). Were recovered 78 COC (RDP:RUP1.68= 3; RDP:RUP1.31= 19; RDP:RUP1.08= 21; RDP:RUP0.83= 35). No significant effect was found in the number of apoptotic cumulus cells (RDP:RUP1,68= 9.33±15.3; RDP:RUP1.31= 40.94±9876; RDP:RUP1.08= 11.12 ±20.0; RDP:RUP0.83= 4.35 ±10.11) and rates cumulus cell apoptotic (RDP:RUP1.68= 0.027±0.02; RDP:RUP1.31= 0.1±0.16; RDP:RUP1.08= 0.12±0.14; RDP:RUP0.83= 0.03±0.1), but there were effect on the percentage of apoptotic oocytes (RDP:RUP1.68= 0%ab; RDP:RUP1.31= 26.32%a; RDP:RUP1.08= 4.76%ab; RDP:RUP0.83= 0%b). The variation in relation between RDP:RUP in diet interfered in apoptosis of oocytes, however, the highest ratio RDP:RUP not different from the two smaller relations.

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A157 OPU-IVP and ET

Effect of the treatment with progesterone and FSH on follicular aspiration of the first follicular wave in ewes

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Keywords: follicle, oocyte, progesterone.

The objective was to determine how progesterone (P4) and FSH of slow release influences the quantity and quality of the oocytes collected by aspiration of the first follicular wave. The experiment was conducted in breeding season (33 º S, Uruguay) in ewes Merino Australian using the Day 0 Protocol for superstimulation of the first follicular wave (Menchaca et al., 2010, Reproduction Fertility and Development, Volume 22, pp 113-118). Intravaginal sponges of medroxiprogesterone acetate (Progespon, Syntex, Argentina) were used for 6 days associated with 125 ug of sodium cloprostenol (Ciclase DL, Syntex) and 200 UI eCG (Novormon, Syntex) given at sponges withdrawal and one dose of a GnRH analogue (8.4 mg of buserelin acetate, Receptal; Hoechst, Germany) was given 36 h after sponges withdrawal. This treatment ensure ovulation (Day 0) and the emergence of the first follicular wave around 72-84h after sponges withdrawal. The ewes were assigned to four experimental groups: Control Group (n=21); P4 Group (n=24) that received an intravaginal device with P4 (0.3 g, DICO, Syntex); FSH Group (n=16) treated with one dose of 80 mg FSHp (Folltropin, Bioniche Animal Health, Canada) reconstituted in sodium hyaluronic acid for slow release (10 ml, MAP-5, Bioniche Animal Health); and P4+FSH Group (n=14) that received the intravaginal device with P4 plus one dose of 80 mg FSHp in MAP-5 solvent. In both progesterone treated groups, the intravaginal devices were kept in place until laparoscopic follicular aspiration that was performed for all the females 72h after Day 0. Statistical analysis was performed by using ANOVA, Kruskall Wallis nonparametric test or chi square test. The treatment with P4 itself analysed as main factor did not affect the number of aspirated follicles (4.1 ± 0.7 vs. 3.8 ± 0.6) neither the number of collected oocytes (2.1 ± 0.7 vs. 1.9 ± 0.4) in comparison with no P4 treated females (P=NS). The treatment with FSH analysed as main effect increased the number of aspirated follicles (6.5±0.7 vs. 4.1±0.7; P<0.05) and the number of collected oocytes (3.4 ± 0.7 vs. 2.1 ± 0.7) in comparison with no FSH treated ewes. An interaction between treatments was observed (P<0,05), and the higher results were obtained in the P4+FSH group. The recovery rate and oocyte quality were not affected in any of the treatments (P=NS). In conclusion, the treatment with intravaginal P4 associated with one im dose of FSH in MAP-5 slow release solvent increases the follicular response and oocyte recovery when aspirating sheep at the first follicular wave.

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A158 OPU-IVP and ET

Effect of camp modulators on *in vitro* pre maturation in production rate and lipid content of crossbred bovine embryos

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Keywords: bovine, forskolin, IVP.

Simulated Physiological Oocyte Maturation system (SPOM; Albuz, Hum Reprod, vol 25, p 12, 2010) was developed to improve the oocytes quality by using cAMP modulators, among which forskolin, which also has delipidant action - desirable for structures directed for cryopreservation. In this system, two stages are proposed: Pre-IVM and extended IVM. The aim of this study was to evaluate the effects of only the pre-IVM step (2h culture in the presence of forskolin and IBMX) on blastocyst rates and lipid content of *in vitro* produced bovine embryos. COCs obtained from slaughterhouse ovaries in three replicates, were selected based on the number of cumulus cells and homogeneous cytoplasm and randomly distributed into two groups: control [C, n = 84; Standard IVM for 24 hours in the commercial medium (Bioklone® Animal Reproduction, Brazil)] and pre-IVM [PM, n = 99; pre-IVM for 2 hours in pre-IVM medium (TCM 199-Hepes, BSA 1,6mg/mL, sodium pyruvate 100mM, ITS 100x, penicillin 10.000UI, streptomycin 10mg/mL, forskolin 100µM and IBMX 500µM) followed by standard IVM]. After IVM, the groups underwent at the same time IVF in TALP and IVC in SOF, both using Bioklone® medium. The cleavage and blastocyst rates were evaluated in D3 and D7, respectively. The blastocysts obtained were fixed in 4% PFA in D7 and stored at 4 ° C until subjected to Oil Red staining technique for lipid content evaluation by analyzing stained area fraction using ImageJ software. The cleavage and blastocyst rates were compared using Fisher's exact test (different averages identified with distinct superscript letters), and the mean lipid stained area fraction compared by Mann Whitney test. Statistical analyzes were performed using GraphPad INSTAT, at a 5% significance level. There was no difference (P>0.05) between the cleavage rates of C and PM (80.95%a - 68 vs 82.83%a - 82), blastocyst rate/total oocytes (39.28%a - 33 vs 28.28%a - 28) and blastocyst rate/cleaved (p = 0.0951; 48.53%a vs 34.14%a) respectively. The mean stained area fraction for lipid to the C was 30.60 ± 5.48 vs 40.23 ± 4.01 for the PM (p>0.05). These results suggest that isolated from SPOM system, usage of pre-IVM step before comercial IVM resulted in no improvement in blastocyst rates, nor the delipidant effect of forskolin was observed in resulting blastocysts.

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A159 OPU-IVP and ET

Effects of sperm selection in single layer Percoll® before freezing on the kinetic characteristics of bovine sperm after thawing

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Keywords: percoll gradient, sperm kinematics, sperm selection.

The aim of this study was to investigate the effects of sperm selection through single layer centrifugation in Percoll® at 90% (SLCP) before freezing, evaluating the spermatic kinetics after thawing through computer-assisted sperm analysis. Semen of 3 Nelore bulls (3 ejaculates of each one), kept in collecting and processing semen station, was collected by artificial vagina. On the day of each collection, after routine sperm evaluation, the ejaculate from each bull was mixed and divided equally at concentration of 1,000 x 106 of sperm (regardless of volume) between the control group (P0, without SLCP) and P9 and P6 groups, which were subjected to centrifugation in 15 ml tubes with 9 ml and 6 ml of Percoll®, respectively. After centrifugation (700 x g/13 min) the supernatant was removed and the pellet of spermatozoa was diluted in freezing medium (tris, fructose, citric acid, egg yolk, glycerol, and antibiotics). Afterwards, 0.5 ml straws were filled with 25 x 10^6 of spermatozoa, cooled for five hours in a cold chamber at 4° C and then frozen in a programmable freezer (Digitcool, IMV, France). After 30 days of storage, four straws from each group and different collection were thawed in water bath at 46º C/20 s and immediately evaluated for spermatic kinetics (CASA, Hamilton Thorne®, Beverly, USA). The following parameters were analyzed: progressive motility (PM; %), curvilinear velocity (VCL; µm/s), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), amplitude of lateral head displacement (ALH; µm), beat cross frequency (BCF, Hz), straightness (STR; %), linearity (LIN; %), and rapid sperm movement (RAP; %). ANOVA and Tukey test were used for analysis of results, with values expressed as mean ± standard deviation, and $P<0.05$ taken as significant. Significant difference ($P<0.05$) was observed between P0 and P9 groups, respectively, for the variables ALH (5.67 ± 0.24 vs. 5.1 ± 0.26), BCF (26.7 ± 0.72 vs. 30.2 ± 1.19), LIN (52.0 ± 2.16 vs. 62.5 ± 2.88) and STR (81.7 ± 2.63 vs. 88.8 ± 1.6). However, no difference was found among all groups for PM, VCL, VSL, VAP and RAP. Results of P6 were similar to those found in groups P0 and P9. Based on the results, SLCP was effective in identifying sperm subpopulations with different kinetics characteristics, which can determine an increase on the rate of fertilized oocytes in *in vitro* embryo production programs. Further analysis in progress, including integrity of acrosome membrane, mitochondrial function, determination of intracellular ROS, membrane lipid peroxidation, and IVF, will provide a better understanding of the proposed protocol.

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A160 OPU-IVP and ET

Cellular and molecular effects of follicular fluid's exosomes from Nelore cows submitted to ovarian superstimulation

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Keywords: exosomes, *in vitro* maturation, ovarian superestimulation.

Cell-cell communication within the ovarian follicle involves many signaling molecules, and this process may be mediated by secretion and uptake of exosomes (Exo) that contain several bioactive molecules. Thus, to gain insight into the effects of superstimulatory treatments in bovine ovary, Exo of follicular fluid from Nelore cows (*Bos taurus indicus*) submitted to ovarian superstimulation by FSH (P36 protocol) and Exo from cows only synchronized were added during *in vitro* maturation (IVM) to assess nuclear maturation, DNA fragmentation and mRNA abundance of bovine cumulus-oocyte complexes (COCs). Nelore cows were slaughtered, fluid from pre-ovulatory follicle aspirated and the Exo were obtained by ultracentrifugation. Five replicates of COCs (20 COCs/group) were matured *in vitro* for 22-24h in TCM 199 supplemented with, BSA, FSH, estradiol, amikacin, pyruvate and: 10% Exo from Non-Superstimulated (Exo-NS group) cows; 10% Exo from P-36 cows (Exo-P36 group) or without Exo (control group). In experiment 1 (Exp.1), after IVM, oocytes were submitted to the TUNEL assay and stained with Hoesch-33342. In experiment 2 (Exp.2), the oocytes and its cumulus cells were submitted to total RNA extraction and reverse transcribed with random primer, separately. The mRNA abundance of H2AFZ and PDE3 in oocytes; GREM1 and COX2 in cumulus cells; and GDF9, BMP15 in both cells types was measured by RT-qPCR using SYBR green system and normalized by the expression of more stable endogenous gene; cyclophilin A (PPIA). The mRNA abundance (target gene/PPIA) was calculated using ΔΔCt method corrected by Pffafl's equation. Effects of the Exo addition on the meiosis progression (%), apoptosis rates (%) and on the mRNA abundance of target genes were tested by ANOVA ($p \leq 0.05$: significant difference and $0.05 < p < 0.10$: considered tendency). In Exp.1, addition of Exo-P36 did not affect apoptosis rates in matured oocytes, nevertheless, Exo-P36 group tended to higher percentage of oocytes in meiosis II (74 ± 3.2) when compared with Exo-NS group (60.2 ± 4.5; $p=0.09$). In Exp.2, no differences ($p>0.10$) on the GDF9, BMP15, H2AFZ, PDE3 mRNA abundance were demonstrated in oocytes, however, GDF9 mRNA abundance tended to be higher in cumulus cells from Exo-P36 group (20.3 ± 13.16) when compared with control and Exo-NS groups (2.2 ± 0.8; 4.1 ± 3.2; $p=0.06$). Even as, BMP15 mRNA abundance also tended to be higher in cumulus cells from Exo-P36 group (5.7 ± 3.2) when compared with control and Exo-NS groups (0.8 ± 0.5; 1.2 ± 1.2; $p=0.06$). In conclusion, the effects of Exo, from cows submitted to P36 protocol, during IVM showed that ovarian superstimulation using FSH seems to modulate the bovine follicular fluid content and consequently it could be used to improve *in vitro* oocyte competence by increasing of meiosis progression and GDF9 and BMP15 gene expression.

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A161 OPU-IVP and ET

Effect of *in vitro* culture on size and sex ratio of bovine embryos on d14 of development

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Keywords: embryos, *in vitro* production, sex determination.

Several studies have been conducted to evaluate the culture effect of in a quality of *in vitro* produced embryos. Most of these studies use as an evaluation method producing blastocyst until the day 7 (D7) or day 8 (D8) of development. Few studies have focused on more advanced stages of embryo, or even if the *in vitro* culture affect embryo development after the embryo transfer into the recipient. Within this perspective, assessment of embryos on day 14 (D14) development allows a more accurate assessment of their quality, produced by different assisted reproduction techniques or those that have undergone different treatments and manipulations. The objective was to evaluate the effects of vitro-produced (IVP) in size and sex of embryos in an advanced stage of development (D14).. For IVP, oocytes obtained from slaughterhouse ovaries were matured, fertilized (D0) and cultured *in vitro* to D7. On D7 culture, blastocysts grade one were selected and transferred in number from 15 to the uterine horn of previously synchronized recipients (group vitro/vivo). As a control, collected embryos on day 7 post-insemination were used of donors overstimulated. After the uterine washing the blastocyst stage and in similar quality *in vitro* were transferred in number from 12 to synchronized recipients (live group vivo/vivo). Embryos from both groups were collected in D14 and measured individually. Later, biopsy of each embryo trophoblast was stored for genomic DNA extraction and determination of sex. The recovery rate of the embryos and sex ratio data male: female from embryos produced *in vivo* or *in vitro* were analyzed by chi-square test ($P < 0.05$). Measurement of embryos was compared using the Kruskal-Wallis test ($P < 0.05$) by Prophet 5.0. We observed a higher rate of embryo recovery for the group vitro/vivo (45.5% ± 55.8) compared to the vivo/vivo group (26.7 ± 55.1). However, when analyzing the proportion of male: female embryos, vitro/vivo (28:19) and vivo/vivo group (12:12), no difference was detected. Relative to the size of the collected embryos in D14, there was no difference between male or female embryos produced *in vitro* (722.6 ± 116.8 and 608.2 ± 121.3, respectively) or *in vivo* (1194.9 ± 244.3 and 1181.5 214.7 ± respectively). Furthermore, the size of the male and female embryos from the same groups was also similar. These results suggest that the developing embryos of D14 are not affected by *in vitro* culture because they suffer no change in the male/female ratio, or the size compared to those produced *in vivo*.

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A162 OPU-IVP and ET

Effect of angiotensin-converting enzyme inhibitor on bovine blastocyst rate

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Keywords: angiotensin, captopril, IVP.

The reproductive performance of herds requires in-depth knowledge of the endocrinology of ovarian reproduction in vivo and *in vitro*. The renin-angiotensin system (RAS) is widely known as regulator of blood pressure, and has been described in several organs and tissues. Captopril is an inhibitor of the angiotensin-converting enzyme (ACE) that prevents angiotensin I (Ang. I) from being converted to angiotensin II (Ang. II). Ang II is capable of reversing the inhibitory effect of follicular cells on the *in vitro* nuclear maturation of bovine oocytes and improves the cytoplasmic maturation of cumulus-oocyte complexes (COC). The aim of this study was to evaluate the effect of captopril on the bovine blastocyst rate. COCs from female cattle originating from slaughterhouses of the municipality of Teresina-PI, Brazil, were used. A total of 472 COCs were distributed into four experimental groups: G1 (n=58) - 0 μM captopril; G2 (n=83) - 5 μM captopril; G3 (n=81) - 10 μM captopril; and G4 (n=78) 15 μM captopril in the maturation medium. After maturation, 300 COCs were co-incubated with spermatozoa at a temperature of 38.5 ºC for 20 h, at 5% CO2. The presumptive zygotes were isolated from the cumulus cells by successive aspirations and washed three times in SOF medium (Nutricell®) supplemented with 5% FBS. Subsequently, they were transferred to a 60 × 15 mm Petri dish containing microdroplets of 100 μL SOF medium (Nutricell®) supplemented with 5% FBS and kept in an oven at 38.5 ºC, at 5% CO2, for seven days. Feeding (exchange of 50% of the SOF medium for a previously-stabilized new one) was performed on the fifth day of growth. The blastocyst rate was evaluated after 168 h (D7) of growth, by the nonparametric chi-square test (X^2). The use of different concentrations of captopril in the growth medium did not change the blastocyst rate, as there was no statistical difference between the experimental groups (G1=37%, G2=48%, G3=53%, and G4=62%). In conclusion, supplementation of the maturation medium with captopril did not improve the blastocyst rate.

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A163 OPU-IVP and ET

Effect of resveratrol on bovine sperm viability in *in vitro* fertilization medium

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Keywords: acrossome, antioxidant, spermatozoa.

Despite a large number of researches about the protector effect of antioxidants on sperm cryopreservation, few studies report their effects on sperm cells for *in vitro* fertilization. The resveratrol (3, 5, 4′-trihydroxystilbene) is a compound found in many species of plants and has several pharmacological activities including antioxidant effect. This study aimed to evaluate the effect of different concentrations of resveratrol diluted in *in vitro* fertilization medium on sperm viability and acrosome integrity. Motile frozen-thawed sperm was thawed at a temperature between 35 °-37 ° C and separated by Percoll gradient method (90 e 45%) and distributed to the following treatments, accordingly to resveratrol concentration in FERT medium: R0 (control – 0µg/mL), R1 (7.5µg/mL), R2 (15µg/mL) and R3 (30µg/mL), and then incubated for 24h at 38.5°C with 5% CO_2 and 95% humidity at concentration of $10x10^6$ spermatozoa/mL. The proportion of live and dead spermatozoa with an intact or reacted acrosome was evaluated by trypan blue-giemsa staining at 6h, 12h e 24h of incubation. Experiments were repeated three times and in triplicate. Data were analyzed by ANOVA and means compared by Student-Newman-Keuls. Values are shown as mean±SEM. The rate of live spermatozoa with decreased from 6 to 24h of incubation in all evaluated concentrations of resveratrol and control, with an accentuated ($P<0.05$) decline between 6h (R0=23.8 ± 1.7%; R1=27.9 ± 2.5%; R2=28.3 ± 1.7% and R3=24.7 ±2.8%) and 12h (R0=7.5 ± 0.7%; R1=9.8 ± 1.4%; R2=9.0 ± 0.6% and R3=7.8 ± 0.7). However, there was no significant difference ($P<0.05$) in the proportion of live sperm among different concentration of resveratrol and the control at 6h, 12h and 24h of incubation. Resveratrol at all concentrations tested increased ($P<0.05$) the percentage of live sperm with reacted acrosome when compared to control, mainly at 6h (R0 = 15.2 ± 1.3%; R1 = 22.9% ± 1.8; R2 = 23.7% ± 1.8; R 3 = 20.9 ± 2.8%) and 12h (R0 = 3.1 ± 0.4%; 6 = R 1, 1 ± 0.6%, R2 = 4.5 ± 0.6%; R3 = 4.8 ± 0.5%) of incubation. In conclusion, the resveratrol concentrations evaluated do not prevent the decrease of sperm viability within 6h to 24h of incubation *in vitro* fertilization medium and increase the number of live sperm with reacted acrosome, showing to have an effect on the integrity of sperm acrosome.

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A164 OPU-IVP and ET

Effect of the length of FSH treatment (4d vs 7d) on the superovulatory response of lactaing Holstein cows

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Keywords: dairy cow, FSH; superovulation.

The aim of the study was to evaluate whether long-term treatment with FSH improves superovulatory response of dairy cows.. Holstein cows (n = 89) producing on average 40.2 ± 5.2 kg/day were randomized to two superovulation protocols at 63 ± 3d DIM, as follows: 1) Short protocol (FSH4d): D-1 = Follicle ablation, D1 to D4 = CIDR insertion and 400 mg of FSH (Folltropin®) applied AM/PM, D3 and D4 AM = PGF2a and CIDR removal together with 2nd PGF2a treatment and 12h before last FSH, D5 AM = GnRH and AI 12 and 24h after GnRH with conventional non-sexed semen from same sire and a single ejaculate. The long protocol (FSH7d) followed a similar sequence of treatments but the 400 mg dose of FSH was extended over 7 days instead of 4. All cows were inseminated by the same AI technician and nonsurgical collection of ova/embryos took place 7 days after GnRH by one of three technicians. Embryo grading was performed by a single technician (blindly) according to IETS guidelines. Data was analyzed by the Glimmix procedure of SAS (version 9.3), the Poisson distribution was assumed for most embryo-related variables and cow was interpreted as the experimental unit. Results for FSH4d and FSH7d were, respectively: total number of CL (FSH4d = 12.0 ± 0.8 vs FSH7d = 12.9 ± 0.8; P=0.22); total structures (FSH4d = 4.8 ± 0.5 vs FSH7d = 4.5 ± 0.6; P=0.49); fertilization rate (FSH4d = 83.8 ± 5.0 vs FSH7d = 92.3 ± 4.0; P=0.18); number of transferable embryos (FSH4d = 1.8 ± 0.4 vs FSH7d = 2.4 ± 0.6; P=0.43); number of freezable embryos (FSH4d = 1.3 ± 0.3 vs FSH7d = 2.1 ± 0.5; P=0.43); percent of degenerated embryos out of fertilized (FSH4d = 41.4 ± 6.2 vs FSH7d = 43.6 ± 6.4; P=0.79). These results suggest that the 7-day (long) FSH protocol failed to improve embryo production in high producing Holstein cows.

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A165 OPU-IVP and ET

Estabelecimento e acompanhamento da g establishment and monitoring of pregnancy in Holstein cows after embryo transfer, produced *in vivo* or *in vitro*

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Keywords: dairy cows, pregnancy, pregnancy loss.

The aim of this study was to evaluate the establishment and maintenance of pregnancy, until parturition, of embryos produced in vivo (superestimulation and uterine flush, SOV-UF) or *in vitro* (ovum pick-up and *in vitro* production, OPU- IVP), derived from donor Holstein cows (black and white Holstein cows, BWH) lactating (LAC) or not (NLAC) and transferred to recipient BWH multiparous (MULT) or primiparous (PRIM). The data was obtained from a commercial dairy farm (Agrindus S/A, Descalvado-SP) during the year 2013. 2225 embryo transfers were performed, and 1337 embryos were obtained from the SOV-UF and transferred to fresh (SOV-UF-F); 474 embryos were obtained from SOV-UF and transferred thawed (SOV-UF-T) and 444 embryos were obtained from OPU-IVP and transferred to fresh (IVP). The pregnancy diagnosis was performed by ultrasonography exams at 30 days of gestation (DG30) and 30 days later the pregnancy was confirmed by rectal palpation (DG60). Pregnancy losses between 30 and 60 days and between 60 days and parturition were also recorded. Data were analyzed using the GLIMMIX procedure of SAS 9.2®, considering category (LAC and NLAC), parity (MULT and PRIM) and embryo production technics (SOV-UF-F, SOV-UF-T, IVP) in the mathematical model, and presented as means of least squares (mean adjusted by the model). The recipients that received embryos derived SOV-UF-F showed higher conception rate at 30 days of gestation than the recipients that received embryos derived SOV-UF-D and IVP [SOV-UF-F: 48.0% (632/1337), SOV-UF-D: 14.3% (64/474), IVP: 31.6% (138/444), P <0.0001], as well at 60 days of gestation [SOV-UF-F: 36.8% (488/1337), SOV-UF-D: 10.7 % (50/474), IVP: 21.8% (99/444), P<0.001]. No significant effects were observed on pregnancy loss between 30 and 60 days of gestation [SOV-UF-F: 21.2% (141/632); SOV-UF-D: 20.8% (14/64); IVP: 30.2 % (39/138); P=0.11] and pregnancy loss between 60 days of gestation and until parturition [SOV-UF-F: 23.7% (120/488); SOV-UF-D: 17.0% (9/50); IVP: 24.9% (25/99); P=0.54]. Additionally, the recipients MULT showed decreased rate of pregnancy at 30 days than PRIM [MULT: 28.9 % (519/1472); PRIM: 33.8% (315/783); P=0.015] and higher pregnancy loss between 60 days of gestation and parturition [MULT: 25.3% (105/392); PRIM: 18.4% (49/245); P=0.049] than the PRIM. Lastly, embryos that were derived from donors LAC showed increased rate of pregnancy loss between 30 and 60 days when compared the embryos derived of donors NLAC [27.5% (122/490) vs. 20.7% (71/343), respectively; P=0.037]. Therefore, it is concluded that pregnancy loss rate was not affected by the type of embryo (in vivo or *in vitro*), however the establishment of pregnancy at 30 and 60 days was higher in embryos produced in vivo and transferred fresh. The number of births affected the pregnancy at 30 days and losses between 60 days and parturition, and lactation status of the donor influenced the pregnancy loss between 30 and 60 days.

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A166 OPU-IVP and ET

Gene expression of AREG and EREG in bovine cumulus cells after *in vitro* maturation in medium supplemented with FCS, BSA or PVA

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Keywords: amphiregulin, epiregulin, transcripts.

The analysis of gene expression of Amphiregulin (AREG) and Epiregulin (EREG) in cumulus cells (CC) showed the importance of these two genes in the regulation of ovulation and CC expansion. This study was carried out to quantify the transcripts of AREG and EREG in bovine oocyte cumulus cells cultured in *in vitro* maturation (IVM) medium, supplemented with FCS, BSA or PVA, as well as in CC of immature oocytes. Follicles (2 to 7 mm in diameter) were punctured from ovaries obtained at slaughterhouse; then, oocytes were divided in two groups, as follows: immature oocytes and *in vitro* matured oocytes. All reagents were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise stated. Oocytes were washed and selected in PBS medium plus 10% FCS (Nutricell®, Campinas, Brazil), but for IVM, groups of 20-25 oocytes were cultured in Medium 199, supplemented with sodium bicarbonate, sodium pyruvate, penicillin, FSH and LH (Lutropin®, Bioniche Inc., Canada), estradiol, cisteamine and different macromolecules (10% FCS, 4 mg/ml BSA or 1 mg/ml PVA), at 38.8º C, under humid atmosphere, 5% CO2, in air, for 24 h. The CC were removed by several pipetting in PBS medium with 0.1% hyaluronidase. Then, the droplets containing only CC were centrifuged (3,355 x g/10 min) and frozen in total RNA extraction medium. Gene expression was investigated by quantitative RT-PCR, normalized by GAPDH constitutive gene; five repetitions were performed for each group. The results were analyzed using ANOVA and Tukey test, with $P<0.05$ taken as significant. The abundance of transcripts for AREG was higher ($P<0.05$) in the CC from *in vitro* matured oocytes, in the presence of FCS, in comparison with immature oocytes and *in vitro* matured oocytes supplemented with PVA or BSA. The CC of immature oocytes had lower expression of EREG compared to oocytes matured *in vitro*. In conclusion, the addition of FCS to IVM medium positively influenced the gene expression of AREG in the CC probably due to its complex composition, acting alone and/or interacting with certain compounds found in the IVM medium.

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A167 OPU-IVP and ET

Identification of arthritis encephalitis caprine (CAEV) in flushing media and embryos from naturally infected herd goats

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Keywords: CAEV transmission, dairy goats, *in vivo* embryos.

The goat production in Minas Gerais state has been growing in recent years, especially with intensive dairy production systems. The artificial reproduction biotechnologies are a powerful mechanism for genetics dissemination. However, the caprine arthritis encephalitis virus (CAEV) is present in most of the dairy goats farms, which represent a risk for disease transmission. The aim of this study is to identify the presence of pro-viral and viral CAEV genome in uterine lavage fluid and embryos samples collected by the non cirurgical transcervical assay. Eight goats were selected with 56.0 ± 13,4kg and a body condition score of 3.5 ± 0.75. The animals were all positive for CAEV in "Western Blotting" diagnosis. Oestrus sycronisation and ovarian superovulation was performed from adapted protocol from Fonseca e*t al,* 2006 (Acta Scientiae Veterinariae, v. 34, sul. 1). After transcervical embryo flushing, embryos that had intact ZP, had their inner cell mass aspirated by a micromanipulator, and the product (zona pellucida and inner cell mass) was stored in RNA later. Centrifugation was performed in uterine liquid washings, until the identification of a pellet (pellet 1) and subsequently viral concentration of uterine flushings (pellet 2) was performed. The material obtained (pellets 1 and pellets 2) was submitted to viral and pro-viral genome extraction with Cador Pathogen® mini kit (Qiagen, Germany) assay. Embryonic samples (zona pellucida and inner cell mass) cDNA synthesis was performed from the viral genome with the use of M MLV enzyme according to protocol. The samples were submitted to nested PCR (Fieni, 2010,). The presence of viral and pro-viral genome in pellet (samples 2), but not in the pellet 1 or embryonic samples (zona pellucida and inner cell mass) was identified. Results shows viral replication could happen in the reproductive system from naturally infected goats, what have never been described before. However, embryos recovery from the flushing media did not show to be permissive to virus. This might indicate that the non cirurgical technique can provide virus dilution, since previous studies in embryos collected surgically revealed the presence of the virus pellet in 1, what did not happen in present work.

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A168 OPU-IVP and ET

Genetics and climatics influence in pregnancy rate in cattle embryos recipients

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Keywords: biotechnology, bovine, pregnancy rate.

Bovine embryo biotechnologies have been widely used in Brazil, mainly in the *in vitro* embryo production (IVEP), and have increased for dairy cattle and European genetic donors (*Bos taurus taurus*; Viana JHV, Embrião, 51:6-10, 2012). However, pregnancy rates in recipients exhibit seasonal variations and in accordance to IVP embryos genetics. The aim of this study was to evaluate the genetic effects of the bull, the donor and the embryo in the pregnancy rates at 30 and 60 days in different seasons. We evaluated data from 2,254 inovulations of IVP embryos generated in the same laboratory, performed over a period of 36 months, in the same property. Crossbred recipients were kept in Brachiaria grass pasture, water and mineral salt ad libitum. Breeders and donors from different breeds were grouped by genetic characteristics, forming the groups Bos taurus taurus (TAU), Bos taurus indicus (IND) and Bos taurus taurus x Bos taurus indicus (MEST). It was considered as summer (VE) months from November to April and winter (IN) months from May to October. The data was plotted in spreadsheets and the pregnancy rates were compared by $\chi 2$ test, considering 5% of probability, using SAS software, version 9.2. Embryos produced with IND bulls semen demonstrated superior results compared to TAU at 60 days (42.0 vs 36.9%; $p<0.05$), however no differences were observed at 30 days. The donor genetic influenced pregnancy rates in both diagnostic periods, whereas the donor TAU showed inferior results (35.8a, 43.2b and 43.7b at 30 days and 31.2a, 41.1b and 40.6b at 60 days in TAU, IND and MEST, respectively; $p<0.05$). In relation to embryo genetics, lowest pregnancy rates ($p<0.05$) were observed in TAU embryos for both diagnostics (32.9a, 43.3b and 43.5b at 30 days and 27.4a, 41.2b and 41.1b at 60 days in TAU, IND and MEST embryos, respectively). The seasons, VE or IN respectively, did not influence pregnancy rates at 30 and 60 days (41.4 vs 42.2 and 38.5 vs 39.6; $p> 0.05$) if considered all embryos. No effects of embryo genetics groups were observed in pregnancy rate on IN months ($p> 0.05$). In VE, TAU embryos showed decreased rates in both diagnostics (34.0a, 44.0b and 43.9b at 30 days and 29.6a, 41.2b and 40.1b at 60 days for TAU, IND and MEST, respectively; $p<0.05$). There was no difference in the occurrence of pregnancy loss between 30 and 60 days in different groups or season ($P> 0.05$). In conclusion, the genetics of TAU animals, regardless of origin from the donor, semen or both, produced embryos with less probability of pregnancy. In general, the seasons of year did not influence the pregnancy rate at 30 and 60 days.

Acknowledgments: Fapemig, Capes e CNPq.

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A169 OPU-IVP and ET

Influence of ovarian status on bovine *in vitro* embryo production

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Keywords: corpus luteum, *in vitro* fertilization, ovaries.

Despite the advances on *in vitro* embryo production, its efficiency remains low. There are evidences indicating that late blastocyst rate depends on the quality of oocytes and sperm cells used as raw material for the process [Dieleman et al., Theriogenology, 57(1), 5-20, 2002]. Regarding oocytes, the source has been considered more relevant for quality than culture conditions in the laboratory (Moussa et al., Anim Reprod Sci, 155, 11-27, 2015). The aim of this study was to evaluate the effect of bovine ovarian status on fertilization and embryo production rates after *in vitro* fertilization. Ovaries were obtained from local slaughterhouse in pairs belonging to the same animal and assigned into three experimental groups according the following criteria: 1) Ovaries with corpus luteum (CL+); 2) ovaries without corpus luteum from cows with cyclic sexual activity (CL-); 3) ovaries from cows in anestrus (NCL) (ovaries without corpus luteum and no visible follicle at divergence or dominance phase in both ovaries). Briefly, after follicular aspiration, groups of 20 cumulus-oocytes complexes (COCs) (420/experimental group) were cultivated for 24 hours at 38.5 °C, 5% CO_2 and saturated humidity in 100 µL of maturation medium (TCM-199/10% of FCS). Afterwards, the COCs were co-cultured with spermatozoa for 18 hours in fertilization medium (SOF/BSA). Finally, presumed zygotes were placed in co-culture for 7 days with cumulus cells under same atmospheric conditions aforementioned. After 18 hours through *in vitro* fertilization process, pronuclei formation was assessed by lacmoid stain. The cleavage rate and blastocyst development were evaluated at 3 and 7 days of *in vitro* culture, respectively. Results were analyzed by Kruskal-Wallis test, using confidence interval of 95%. There was no difference in pronuclei formation between experimental groups. Cleavage rate of embryos derived from oocytes obtained from ovaries of cows in anestrus (NCL) was lower [49.5% ± 3.6, (p=0.029)] compared with CL- group (62.2% ± 3.9). Nevertheless, there was no difference in the cleavage rate of embryos derived from oocytes collected from ovaries with (CL+) and without (CL-) corpus luteum from cows with cyclic activity. In addition, there was no difference on blastocyst production rate between NCL, CL- and CL+ groups (20.3% ± 3.3, 20.9% ± 3.2 and 16.4% ± 2.9, mean ± SEM, respectively). In conclusion, the source of oocytes could influence the rate of cleavage after *in vitro* fertilization. However, under the culture conditions assessed, there was no effect on blastocyst production.

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A170 OPU-IVP and ET

The animal temperament influence on responsive rates to hormonal protocol and pregnancy in recipient cows of embryos

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Keywords: hormonal protocol, pregnancy, temperament.

The aim of this research was to study the influence of animal temperament in the response to hormonal synchronization protocol for embryo transfer in fixed-time (TETF) and in pregnancy in embryos recipient cows. It were used (n = 235) embryos recipient cows, native from Acre, Brazil, whit uni or multiples pregnancies, 03 to 06 years old, not-lactating, extensive raising, with ad libitum water and mineral salt. The hormonal synchronization procedure was based on simplifying P36 TETF protocol, as follow: on a random day of the estrous cycle (D0), each cow received 1g P4 intravaginal progesterone device and 2.5 mg estradiol benzoate (BE) intramuscular (IM). On D8, P4 implant was removed and 150μg D-cloprostenol (PGF2α), 400 IU eCG and 1mg BE were administered IM. On D16, one embryo (blastocyst grade 1 or 2) was transferred to each cow after the detection of one corpus luteum (CL) in one of the ovaries (US - Aloka SSD 500, Aloka, Japan). Embryos were obtained by *in vitro* production technique (PIV) originating from dairy Gir donors and bulls. Temperaments were classified in three levels: 1 - docility, 2 - slightly aggressive temperament and 3 - very aggressive temperament, according to cow's behavior during the synchronization process until embryo transfer'day (D16). Chi-square test was used in this study. From 235 cows submitted to hormonal protocol (TETF), 77 (33%) showed no CL in the ovaries by ultrasound examination at D16, therefore classified as unresponsive to hormonal protocol. From those, 53 cows (69%) were level 1, 16 (21%) level 2 and 8 (10%) level 3. On the other hand, 158 (67%) cows responded to the protocol, presenting a CL and been submitted to TETF. From 158 transferred embryos, 54 (34%) resulted in pregnancies. The response or not to hormonal protocol showed no statistical difference in relation to temperament levels. However, in relation to pregnancy rate, group 1 animals (docility) had higher levels (41%) than groups 2 and 3 (21% and 28% slightly and very aggressive, respectively). Therefore the recipient temperament didn't influence significantly the hormonal protocols response ($P > 0.05$), but influenced the pregnancy rate ($P < 0.05$).

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A171 OPU-IVP and ET

Inhibition of methylation with 5-aza-2'-deoxycytidine interferes on development of bovine embryos derived from heat-shocked oocytes

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Keywords: IVEP, methylation, stress.

Epigenetic modifications are required during pre-implantation embryo development but little is known about the effects of heat shock. Chemical agents that modulate epigenetic events can contribute to understand the effect of IMV and heat shock on embryo development. This study evaluated the effect of 5-aza-2'-deoxycytidine (5-aza; Sigma, St. Louis, USA), a DNA methylation inhibitor, on development of bovine embryos derived from oocytes submitted or not to heat shock during IVM. Experiment 1 (NHS – non-heat-shock) evaluated the effect of 5-aza on development of embryos derived from oocytes matured at 38.5°C and 5% CO_2 for 24h, and the Experiment 2 (HS - heat shock) evaluated the effect of 5-aza on development of embryos derived from oocytes matured at 41.5ºC for 12h in 6.5% CO_2 followed by 38.5°C for 12h in 5% CO2. After IVM and IVF, denuded presumptive zygotes were cultured with 0 or 10 nM of 5-aza for 24h or 48h in CR2aa plus 2.5% FBS at 38.5°C with 5% CO_2, 5% O_2 and 90% N_2. After that, embryos were cultured in CR2aa plus 2.5% FBS until day eight post-fertilization. The experiments were composed by the following treatments: Exp. 1: NHS (control, without 5-aza; n=391); NHS24h (5-aza for 24h, n=380) and NHS48h (5-aza 48h, n=379); Exp. 2: HS (control without 5-aza; n=329); HS24h (5-aza 24h, n=320) and HS48h (5-aza 48h, n=381). The proportion of embryos that reached the 8-cell stage on day three (D3) post-fertilization was analyzed by chi-square. Total cleavage rate on D3 and blastocyst rate on day eight (D8) were analyzed by ANOVA and means compared by SNK. Values are shown as mean±SEM. The proportion of embryos with 8-cell in the Exp 1 was lower ($P<0.05$) in NHS24h (28.1%) and NHS48h (33.4%) than in the control (NHS: 42,3%). In Exp. 2 HS48h (23.0%) had lower ($P<0.05$) proportion of embryos at 8-cell stage than the control (HS: 34.0%), with no significant difference with HS24h (27.1%). There was no ($P>0.05$) difference in the cleavage rate among treatments in the Exp. 1 and in the Exp. 2. In the Exp. 1 blastocyst rate was lower ($P<0.05$) for NHS48h (15.7 ± 2.9%) than for NHS (32.2 ± 3.4%) and NHS24h (25.8 ± 3.9%) treatments, and in Exp. 2 blastocyst rate was lower ($P<0.05$) for HS24h (9.5 ± 2.2%) and HS48h (11.1 ± 2.4%) than for HS (21.6 ± 3.4%). In conclusion, inhibition of DNA methylation for 48h in embryos derived from non-heat-shocked oocytes influences the production of blastocysts (Exp. 1) whereas that same effect is found with shorter time of embryo exposure to DNA methylation inhibitor (24h) for embryos derived from heat-shocked oocytes (Exp. 2). Those data suggest that embryos derived from heat-shocked oocytes are susceptible to epigenetic modulation but in a different time-dependent manner from those derived from non-heat-shocked oocytes.

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A172 OPU-IVP and ET

Hormonal stimulation in Nelore calves for *in vitro* embryo production

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Keywords: hormonal stimulation, IVF, prepubertal females, OPU.

The study of reproductive management on prepubertal bovine females for including these animals in the IVF could be an alternative for accelerating genetic gain by reducing the generation interval. This work aimed to evaluate a hormonal treatment response on the ovarian characteristics and on the OPU (Laparoscope Storz Xenon300W. Tuttlingen, Germany)/IVF in 9 Nelore calves (cross-over design) 4-7 months old. In the control group (CG, n=9) transrectal ultrasonography (US) was performed daily (MyLab 30VetGold,Esaote, 5-7,5MHz transducer. Génova, Italy) during 8 days (D0 to OPU) and ablation of larger follicles was performed on D2 (5 days before OPU). In the treated group (TG, n=9) US was also performed during 8 days, which D0 represented the start of treatment (Intravaginal device. Progesterone-P4 0.33g. Eazi-Breed-CIDR, Pfizer Animal Health, Brazil) including an injection of estradiol benzoate (im. 2mg. Ric-BE, Tecnopec-Brazil). From the D4 on, 6 FSH injections were administered, during 3 days (im. 12/12h: 40mg+5 20mg doses. Total:140mg; Folltropin, Bioniche Animal Health, Belleville-Ontario, Canada). On the last FSH dose, it was administered 2.5mg of LH (im. Lutropin, Bioniche Animal Health, Belleville-Ontario, Canada). The OPU was performed 20-24h after the last FSH injection (D7). The P4 devices were removed right after the OPU. Aspirated COCs were selected, matured, fertilized and cultured *in vitro* until D7. As IVF control, COCs obtained from slaughterhouse ovaries were used (Slaughterhouse Group-SG). The US or OPU grouped data were simultaneously compared between CG vs TG on D0 and D7 and analyzed according to their distribution, by t and Man-Whitney tests. The IVF results were compared among CG, TG and SG by Chi-square test and Kruskal Wallis (PROPHET 5.0, BBN-Systems&Technologies, Cambridge-Massachusetts, USA). The TG had increased ($p<0.05$) total follicular population (20.0 ± 4.95, D0 vs 26.66 ± 4.24, D7), follicles ≥2,5mm (4.11 ± 1.96, D0 vs 11.55 ± 4.09, D7), ovarian diameter (13.08 ± 1.0 mm, D0 vs 14.81 ± 1.38mm, D7) and the number of aspirated follicles (95 vs. 152, respectively CG vs TG). The TG had more grade I and II oocytes (59% vs 25% in TG vs CG, respectively) and, conversely, the CG had more grade III and IV oocytes (53.3% vs 37.1% in CG vs TG), $p<0.05$. However, it did not increase ($p>0.05$) the cleavage rate (49.33% vs 51.42%, respectively CG vs TG), the rate and the number of blastocysts in D7 (1.33% vs 8.57% or 3 vs 9, respectively for rate or number in CG vs TG. SG blastocysts rate: 24.41%) nor the number of embryos/female donor (0.33 vs 1.0, respectively CG vs TG). It was concluded that this hormonal treatment increased the number of observed and aspirated follicles, grade I and II oocytes, but it did not increase embryo production on these animals.

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A173 OPU-IVP and ET

Postpartum spontaneous ovulation in quarter horses embryo recipients mares

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Keywords: ET, follicles, ultrasonography.

The seasonal polyestrous is characteristic of the equine species, which enters in heat regularly in the period of greatest brightness. In spring and fall transitional period, the follicular growth is still uneven and determines failures in ovulation, by the negative effect of melatonin on the neuroendocrine system. In embryo transfer (ET) programs, the inclusion of mares, as recipients, in early spring determining limited success. Therefore, this study evaluated the influence of the foaling period on first estrus post partum, time to first ovulation and follicle diameter of embryo recipients. Quarter Horse mares (n = 34) with 11 y.o. in average, and 500-600kg were evaluated from February to March 2015 in a farm located in Weatherford, Texas/USA. The mares received alfalfa and sweet feed, and were kept in paddocks after the second day postpartum (d.p.p.). The mares were distributed according to the birth date on A (Feb 1st to 28th), B (March 1st to 15th) and C (after March 15th). After the 8th d.p.p., the mares were evaluated (every other day) by transrectal palpation and ultrasonography for the following parameters: time of foaling, follicular development, diameter of the pre-ovulatory follicle, ovulation distribution in right or left ovary, d.p.p. after first ovulation, and the follicles growth rate. Once detected, a follicle with diameter ≥30mm was followed until ovulation. The data were submitted to PROC GLIMMIX of SAS® (SAS 9.3, USA, 2003). The total number of ovulations on the left and right ovary were 21:13, respectively. On the left ovary the growth rate was 5.62 ± 0.69mm/day, the ovulation occurred with follicular diameter of 43.57mm ± 0.99 to 12.19 ± 0.43 d.p.p. On the right ovary, the growth rate was 3.36±0.4 5mm/day and ovulation occurred at 13.07 ± 0.8 d.p.p. with a diameter of 43.00 ± 1.13mm. All mares (n=8) at group A ovulated in the left ovary with a growth rate of 6.7 ± 1.07mm a day and in average ovulated at 11.2 ± 0.65 d.p.p. with a follicle diameter of 43.1 ± 1.6mm. Group B, showed 7 ovulations in the left ovary, and daily growth rate was 4.00 ± 1.30mm/day. Ovulation occurred 12 ± 0.61 d.p.p., and the average follicular diameter was 42.33 ± 1.45mm. In the right ovary 6 ovulations occurred, the growth rate was 3.00 ± 0.57mm/day, ovulation occurred after 12 ± 1.03 d.p.p., and the mean follicular diameter was 40.00 ± 1,87mm. Group C showed 6 ovulations in the left ovary with a growth rate of 5.75 ± 1.03mm/day, ovulating on day 13.66±0.8 with a diameter of 45.33 ± 2.17mm. Seven ovulations occurred in the right ovary; the growth rate was 3.57 ± 0.64mm/day, until ovulation at 14 ± 1.15 d.p.p. with a diameter of 45.14 ± 0.73mm. The analysis of time of foaling revealed no difference in time to p.p. ovulation (P=0.717), daily growth rate (P=0.741), and the follicle diameter at ovulation (P=1.00). The time of foaling did not influence the follicular growth rate, postpartum days to ovulation or follicular diameter of recipient mares prepared for ET that foaled at different dates in the season.

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A174 OPU-IVP and ET

In vitro production parameters in Sindhi breed bovine females (*Bos taurus indicus)*

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Keywords: *Bos indicus*, IVF, OPU.

Ultrasound-guided follicle aspiration (OPU) related to IVP of embryos is a technique of great economic interest that have been growing rapidly in the industry due to the efficiency of sex-sorted semen, and zebu cattle (Bos indicus) have been largely used for dairy purposes (Viana et al., Theriogenology, 73, 966-972, 2010). Therefore, the aim of this study was to evaluate the effect of the donor age, season of the year, sire and type of semen (sexed and non sexed) on IVP of Sindhi embryos. Data were provided by Sexing Technologies do Brazil (Sertãozinho, São Paulo, Brazil) regarding to 499 OPU sessions performed from January 2008 to January 2015 on 150 Sindhi oocyte donors raised at different farms from São Paulo state. Conventional and sex-sorted Sindhi semen from 22 different bulls were used. The effects of the donor (150 animals), age of the donor (up to 6 years vs. over 6 years old), season of the year (rainy vs. dry), sire (22 animals) and type of semen (conventional vs. sex-sorted) on variables like number of oocytes collected, number of viable oocytes (in grades I, II and III), number of degenerated oocytes, cleavage rate and blastocysts rate in D7 were analyzed. Data were analyzed by ANOVA using the GLIMMIX procedure of SAS. All OPU sessions resulted in 7.971 COCs recovered. The season of the year (rainy vs. dry) did not affect the number of oocytes collected per OPU (23.4 vs. 24.0 ± 1.43; P=0.74). However, in the rainy season the number of viable oocytes was greater (67.8 vs. 64.0 ± 0.01%; P=0.02), and the number of degenerated oocytes smaller (32.2 vs. 36.0 ± 0.01%; P=0.02). However, the cleavage and blastocyst rates did not differ (P>0.45) in OPU performed between the different seasons and they were in average 72.0 ± 0.03 and 26.8 ± 0.01%, respectively. The age of the donor at the time of OPU affected the results of PIV. Donors with up to 6 years old had greater number of oocytes collected (26.9 vs. 20.8 ± 1.33; P<0.01), as well as greater number of viable oocytes (68.5 vs. 64.4 ± 0.01; P<0.01) and smaller number of degenerated oocytes (31.5 vs. 35.6 ± 0.01; P<0.01). However, there was no difference on cleavage rate between young and old cows (74.4 vs. 70.0 ± 0.03%; P=0.31), nonetheless there was a tendency for young cows to had smaller blastocyst rate in D7 (25.0 vs. 28.2 ± 0.01%; P=0.07). The conventional semen resulted in better cleavage rate (76.4 vs. 58.9±0.04%; P<0.01) and blastocyst rate than the sex-sorted (27.5 vs. 23.1 ± 0.01%, respectively; P=0.02). There was an effect of sire on the number of oocytes collected (P<0.03) and on blastocyst rate (P<0.02). In addition, there was an effect of donor on viable oocytes (P<0.02), in the number of degenerated oocytes (P<0.02), and on blastocyst rate in D7 (P<0.01). In conclusion, in donors with less than 6 years old, the number of oocytes collected and embryo produced is greater. The sexed semen is less efficient than the conventional one in IVP programs in this breed. Females Sindhi produce a greater number of viable oocytes during the rainy season, when the availability of forages is greater.

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A175 OPU-IVP and ET

Plasma FSH profile following single injection of pFSH combined with hyaluronan

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Keywords: FSH, Holstein heifers, plasma profile.

We evaluated the plasma FSH profile after injection of different doses of porcine FSH (pFSH; Folltropin®, Bioniche) associated with hyaluronic acid (HA; MAP-5®, Bioniche). A total of 23 Holstein heifers were allocated in one of four groups: Control (no superstimulatory treatment; n = 4), F200 (200 mg of pFSH, n = 6), F200HA (n = 6) and F300HA (n = 6; 200 or 300 mg of pFSH diluted in HA 0,5%). On a random stage of the estrous cycle, all heifers received a Norgestomet ear implant (Crestar® MSD), 2.0 mg IM of estradiol benzoate (RIC-BE®, Tecnopec) and 0.150 mg of cloprostenol (ESTRON®, Tecnopec). On Day 5, all females received 0,150 mg of cloprostenol and on Day 7 they were submitted to an ultrasonographic evaluation to verify the diameter of the largest follicle. On Day 8 and Day 9 the F200 group received 200 mg of pFSH (14.3 mg/mL) administered in 4 decreasing doses (57.1, 57.1, 42.9 and 42.9 mg) every 12 h. The F200HA and F300HA groups received a single dose of pFSH (IM) on Day 8 AM, 5.0 ml (F200AH) and 7.5 ml (F300HA; 40mg/ml of FSH). The control group received no additional treatment. On Day 12 AM, the implant was removed. Blood sampling started on Day 8, immediately before the first pFSH administration (0h). The samples were collected by jugular vein every 6 h until 96 h to evaluate the plasma FSH concentration. The variables were analyzed by orthogonal contrast using the GLIMMIX procedure of SAS. The established contrasts were: C1 (superstimulation effect): control vs. (F200 + F200HA + F300HA); C2 (HA effect): F200 vs. (F200HA + F300HA); C3 (dose effect): F200HA vs. F300HA. The area under the curve was calculated by the trapezoid method. The total period with elevated FSH concentration (greater than two standard deviations) was determined by the interval between the treatment and FSH return to basal levels. Heifers submitted to superstimulatory treatment (F200: 75.8 ± 5.6; F200HA: 64.8 ± 11.5; F300HA: 97.8 ± 5,3 ng*h/mL) presented higher area under the curve when compared to the control group (53.8 ± 5.1 ng*hr/mL; C1, P = 0.002). The F200 group did not differ (C2; P = 0.56) from females treated with HA and; F300HA group animals presented higher area under the curve in comparison to F200HA group (C3; P = 0.006). Still, F200 group presented an extended period with high plasma FSH concentration (55.0 ± 4.5h) compared to groups receiving single dose of pFSH in HA (C2; F200HA: 32.0 ± 8.2 and F300HA : 37.0 ± 4.5h, P < 0.0001), being this period similar among the groups treated with HA (C3, P = 0.17). Therefore, the treatment with a single dose of pFSH diluted in HA increased plasma FSH concentrations compared to the Control group, being similar to the treatment with two daily injections. However, treatment with pFSH diluted in HA presented shorter period with high plasma FSH concentration compared to the group with pFSH daily injections.

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A176 OPU-IVP and ET

GnRH potential to synchronize follicular emergence and ovulation prior to superovulatory day 0 protocol in sheep

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Keywords: lecirelin, MOET, superovulation.

The role of GnRH to synchronize ovulation and follicular emergence previous to superovulatory protocol, started on the first day of the estrous cycle (Day 0), was assessed in Santa Inês ewes. For estrus synchronization, 60 mg medroxyprogesterone acetate sponges were used for 6 d plus 37.5 µg d-cloprostenol and 300 IU eCG at fifth day. After sponge removal, ewes were assigned to three treatments: GControl – saline at 12 h (n = 10); G24h – GnRH at 24 h (n = 10); or G36h – GnRH at 36 h (n = 9). Ovarian ultrasonography was conducted every 12 hours, after sponge removal, to assess the occurrence of ovulation and emergence of follicular waves until the fifth day of the estrous cycle. Ewes from G24h and G36h had earlier ovulation (48.0 ± 3.2 and 56.7± 1.9 h) compared to GControl (64.1 ± 3.0 h – $P < 0.05$). It is reasonable to affirm that G36h was more effective in synchronizing ovulation compared to G24h probably due to the lower SEM obtained. The follicular growth in the post-ovulatory day was affected by day of the estrous cycle ($P < 0.05$) as well as by interaction treatment x day of the estrous cycle ($P < 0.05$). There was a greater population of medium follicles during the first 24 h post-ovulatory period in G24h compared to GControl and absence of large follicles in G36h between 36 and 72 h after ovulation. The greatest population of medium follicles in G24h compared to GControl may arise from the previous ovulatory follicular wave, but due to anticipation of the LH surge, it was not able to promote the growth and maturation of these follicles. After 60 h, the medium follicles from wave emergence of GControl and G36h stabilized compared to the follicles from G24h. The greater number of dominant follicles 12 h after ovulation in G36h compared to G24h has correlation with the largest number of ovulated follicles in this group. It is important to highlight that during the first 96 h of the estrous cycle, G36h presented no dominant follicle between 36 and 72 h after the ovulatory period. In conclusion, considering the beneficial effects of G36h in synchronizing ovulation and to promote the absence of dominant follicles in the first days of estrous cycle. According to data obtained, the best time to start the superovulatory treatment, known as "Day 0", could be 80 h after sponge removal (56 h for the occurrence of ovulation plus 24 h to reset dominant follicles), in the induction of synchronized estrus, for Santa Inês ewes.

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A177 OPU-IVP and ET

Bovine and buffalo *in vitro* embryo production with the addition of lippia origanoides essential oil in the maturation medium

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Keywords: antioxidant, buffalo, *in vitro* production of embryos.

The use of embryo *in vitro* production (IVP) has grown in recent years, however, the efficiency of the process is low with unsatisfactory embryo production levels. It is believed that many factors can influence this process, including, reactive oxygen species (ROS) produced as a result of artificial maturation and culture conditions. Thus, antioxidants have been incorporated into the IVM and IVC medium in attempt to reduce the formation of ROS and to improve the embryo production rates. A natural antioxidant already used in cell culture, whose active ingredient were carvacrol and thymol, extracted from Lippia origanoides plant, represents a promising potential to be used in embryo culture (Vicuña, et al., Fitoterapia, v. 81, p.343-349, 2010). Thus, the aim of this study was to evaluate the effect of supplementation of maturation medium for buffaloes and cattle IVP with essential oil of Lippia origanoides (EOLO) at different concentrations. Were used 2052 bovine oocytes and 1026 buffalo oocytes recovered from slaughterhouse ovaries, divided into 5 treatments consisted of: T1 (Base Media (BM): TCM 199 + 10% FBS + 22μg/ml pyruvate + 5UI /mL LH + 0,05μg/ml FSH + 1μg/ml estradiol + 83.4 μg/ml amikacin), T2 (BM + 50 μM/ml cysteamine), T3 (BM + 2,5μg/ml EOLO), T4 (BM + 5 μg/ml EOLO) and T5 (BM + 10 μg/ml EOLO). The reagents used were acquired from Sigma-Aldrich®, St. Louis, USA. Oocytes were maturated at 5% CO_2, 38.5 °C for 24 hours. IVF occurred for a period of 18-20 hours and then the zygotes were denuded and cultured in SOF medium + 2.5% FBS for 7 days. It was evaluated the rate of cleavage after 48 hours of cultivation and the production of blastocysts on 7 and 8. For statistical analysis we used the ANOVA test and considered statistically significant $p < 0.05$. The cleavage rate (mean ± standard error) did not differ ($P>0.05$) between treatments for buffaloes (39.87 ± 5.54; 35.64 ± 5.60; 44.16 ± 17.04; 45.19 ± 5.56 and 43.57 ± 6.86; respectively for treatments T1, T2, T3, T4 and T5). The blastocyst rate for buffaloes was also similar ($P>0.05$) between treatments (30.21 ± 6.49; 19.52 ± 5.92; 27.56 ± 5.11; 32.87 ± 6.49 and 23.35 ± 4.77; respectively for treatments T1, T2, T3, T4 and T5). For cattle, cleavage rates did not differ ($P>0.05$) between treatments (64.06 ± 3.98; 62.65 ± 3.59; 64.72 ± 4.04; 53.32 ± 3.84 and 65.51 ± 4.90; respectively for treatments T1, T2, T3, T4 and T5). The cattle blastocyst rate did not differ $P>0.05$ between treatments and was 27.25 ± 2.98; 26.82 ± 3.94; 24.97 ± 3.52; 23.36 ± 2.57 and 27.14 ± 2.87 for T1, T2, T3, T4 and T5 ($P> 0.05$), respectively. The use of Lippia origanoides essential oil did not affect cleavage and development capacity of cattle and buffaloes in vitro produced embryos.

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A178 OPU-IVP and ET

In vitro production of bovine embryos and buffalo during two periods of the year

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Keywords: *Bos taurus,* buffalos, *in vitro* production of embryos.

The PIVE aims to maximize the genetic gain by increasing the number of female offspring during the reproductive life and optimizing the use of semen dose. Despite the considerable importance of this biotech, the rates found in the literature are variable, especially when it comes to bovine and buffalo. It has been reported that the blastocyst production rate in buffalo ranges around 20%while in the bovine it is around 48% (Elamaranetal, Reprod. Dom. Anim, V.47, p. 1027 to 1036.2012). One of the possible reasons for the low production of embryos in buffaloes is the influence of seasonality on the reproduction because they are considerate short days animals. In view of this, the present study aimed to evaluate the *in vitro* production of bovine embryos and buffalo during two periods of the year, considered favorable and unfavorable to buffalo. The experimental period was divided into favorable season (1 March to August 31) and unfavorable (1 September to February 28) according to the seasonality characterized for buffaloes. Altogether 174 and 566 buffaloes and cattle oocytes were used, respectively. They were placed in wash medium comprising TCM 199, 10 % fetal bovine serum, 22µg / ml sodium pyruvate and 83µg / ml of amikacin sulfate. Oocytes were matured in an incubator (38.5 ° C , 5 % CO_2 , 95 % humidity) for 24 hours in solution (TCM 199 + 10 % FCS + 22µg / ml + Pyruvate 5UI / ml LH + 0.05 mg / ml FSH + 1 / ml estradiol + 83.4 ug / ml amikacin + 50 mcg / ml cysteamine). They were fertilized for 18 to 22 hours and the sperm used was from the same bull with fertility proven in PIVE. The reagents used were from Sigma - Aldrich®, St. Louis , USA . The presumptive zygotes were denuded and then cultured in SOF medium (Synthetic oviduct fluid) + 2.5 % FBS for 7 days. Cleavage rate was assessed 48 hours after fertilization. The production of blastocysts was evaluated on D7 and D8. For statistical analysis, the Shapiro -Wilk test was used to assess the normality of continuous variables. For the mean comparison we used the Student T- test test and the level of significance was $p < 0.05$. Regarding the time of year (favorable and unfavorable) there was no difference between the cumulus expansion rates (89.49 ± 6.61 vs 92.00 ± 2.83) cleavage (55.87 ± 4.22 vs 62, 83 ± 4.20) and blastocyst formation in cattle (20.12 ± 6.33 vs 27.68 ± 4.18) ($p> 0.05$). In Buffalo there was no difference between the cumulus expansion rate (78.73 ± 4.44 vs 77.67 ± 7.64) , cleavage (36.29 ± 4.11 vs 35.13 ± 10.12) and blastocyst formation (22.79 ± 7.14 vs 16.90 ± 9.58), in both favorable and unfavorable time, respectively. In the favorable time there was no difference in cumulus expansion and blastocyst production among cattle and buffaloes ($p> 0.05$). However the cleavage rate was differed between bovine and buffalo ($p <0.05$) in unfavorable time.

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A179 OPU-IVP and ET

In vitro embryo production of Nelore (*Bos indicus*) donors submitted to a commercial program during 12 months

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Keywords: bovine, embryo, IVF.

The aim of this study was to evaluate the effect of the number of OPU sessions (OPU), bull and season (summer vs. winter) on the *in vitro* embryo production (IVEP) of Nelore (Bos indicus) donors submitted to a commercial IVEP program during 12 months. Data of 36 cycling cows were analyzed during 12 consecutive follicular aspirations (OPU), with a 30 days interval at an experimental farm (Instituto de Zootecnia Sertãozinho, APTA). All cows were aspirated without previous synchronization of the follicular wave. A total of three Nelore bulls were used for *in vitro* fertilization of retrieved oocytes. Data were analyzed as repeated measures using the GLIMMIX procedure, SAS 9.3. The total number of oocytes [20.3 ± 2.7 (1st session) vs. 13.5 ± 1.21 (12th session); P = 0.0003] and number of viable oocytes [17.0 ± 2.4 (1st session) vs 10.0 ± 1.0 (12th session); P = 0.001] decreased over time according to the number of aspiration sessions. It was observed interaction (P = 0.01) between the number of OPU sessions and season for rate of viable oocytes, which was greater during the winter (P = 0.002) in sessions 1, 2, 3, 6, 10, 11 and 12 (Winter: 86.4, 83.3, 77.1, 76.5, 74.8, 76.3, 75.5, 74.0, 69.8, 77.6, and 80.1 78.4% and Summer: 64.7, 67.3, 72.0, 77.9, 76.7, 67.5, 67.2, 76.4, 71.8, 74.7, 68.8 and 69.4%, aspiration sessions 1 to 12, respectively). There was no effect of aspiration session on the number of embryos produced by OPU session (P = 0.97) and blastocyst rate (P = 0.30). The total number of oocytes (P = 0.29) and number of viable oocytes (P = 0.11) did not differ between season. However, the number of embryos per OPU session (6.1 ± 0.4 vs 4.5 ± 0.3; P = 0.06) and blastocyst rate (34.86 vs 31.86%, P = 0.009) was greater in winter compared to summer. The number of embryos per OPU session was not different among the diferent bulls (P = 0.14). However, a bull effect was observed on blastocyst rate (Bull A: 31.0%b; Bull B: 30.3% b and Bull C: 40.4% a; P = 0.0004). In conclusion, the number of total and viable oocytes decreased over time, according to the consecutive OPU procedures. Still, the efficiency of in vitro embryo production in Bos indicus donors can be compromised during the hot season of the year. Finally, the efficiency on in vitro embryo production can be affected according to the bull used for IVF.

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A180 OPU-IVP and ET

Acrosome reaction induced by theophylline associated or not to heparin in bovine semen

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Keywords: capacitation, fertilization, sperm.

The sperm capacitation consists in biochemical and physiological changes during which the sperm becomes hyperactivated and able to undergo the acrosome reaction to penetrate the zona pellucida of the mature oocytes. The aim of this study was to evaluate theophylline as a capacitation inducing agent in replacement or in combination with heparin for sperm cells acrosome reaction. Theophylline (T1633 - Sigma-Aldrich®) was added to fertilization medium provided by Biodux® company (Campinas, SP, Brazil). The experiment was performed with 4 Gir breed bulls and 3 treatments, in a total of 12 experimental groups. Each bull was evaluated using the following treatments: Treatment 1 (T1): Heparin - 10µg/mL; Treatment 2 (T2): Theophylline – 5 mM; Treatment 3 (T3): Heparin (10µg/mL) + Theophylline (5mM). Heparin is used as capacitation agent in most of embryo production laboratories, and for this reason was chosen as the control group. The acrosome integrity was analyzed by double staining technique (Trypan blue/Giemsa – TBG) described by Didionet al. (Gamete Res, v.22, p. 51-57, 1989). The semen was thawed and submitted to Percoll gradient separation (45 and 90%). Tubes containing fertilization T1, T2 and T3 medium were inseminated with 2 x 10^6 sperm/mL and kept on incubator at 38.8°C and 5% CO_2, in the absence of oocytes. The semen was incubated in capacitation medium for 0, 6, 12 and 18h then stained with Trypan blue / Giemsa and analyzed by bright field microscopy with 1000x magnification to evaluate the acrosome reaction. The analyzed characteristics in sperm cells were true acrosome reaction - Acrosome and post-acrosome region unstained; False acrosome reaction - Acrosome unstained and post-acrosome region stained; Dead - Stained post-acrosomal region and acrosome staineds. The data were submitted to analysis of variance followed by Tukey Kramer test ($p < 0.05$). Treatment did not affect the acrossome reaction analysis. The same was observed to bulls. However, the true acrosome reaction rate was higher ($p < 0.05$) at time 0h (61.50 ± 6.78) compared to 6h (19.63 ± 12.71), 12h (7.21 ± 4 40) and 18h (7.04 ± 2.66). For the dead sperm, we observed a higher rate ($p < 0.05$) at time of 12h (84.46 ± 5.82) and 18h (86.75 ± 4.19). It was observed that the incidence of true acrosome reaction was relatively lower than the dead sperm rate, which may be due to the total incubation time (18 hours). This suggests that the incubation conditions in the absence of COCs and essential growth factors for the acrosome reaction stimulation impaired sperm viability in vitro. However, theophylline was as effective as heparin in the induction of acrosome reaction.

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A181 OPU-IVP and ET

Efficient recovery of oocytes from ''onça parda'' (*Puma Concolor*) by laparoscopic ovum pick-up of gonadotropin-stimulated females

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Keywords: laparoscopic ovum pick-up, oocytes, *Puma concolor*.

Developing of an optimized procedure for oocyte collection would be critical for allowing the use of *in vitro* embryo production technologies (IVEP) and somatic cell nuclear transfer (SCNT) as tools for rebuilding balance in animal numbers for such endangered species. One young adult puma female of 2.5 years and two sister juvenile females of 1.5 years of age, were injected with 750 UI eCG five days prior to laparoscopic ovum pick-up (LOPU). The puma adult and one of the young females were also injected with 500 IU of hCG 24-30h prior to LOPU to promote *in vivo* maturation of oocytes, while the other sister female could not be injected and served as control. All injections were conducted using blow darting technique. Animals were deprived from food (24h) and water (12h) in preparation for surgery.The LOPU procedure was similar to the one previously described in small ruminants. Briefly, the females were restrained on a laparoscopy table in a 45° angle and then, using a 5 mm laparoscope and an atraumatic grasping forceps to uncover the ovaries, all follicles ≥ 2 mm diameter were aspirated using a 20G needle mounted in a plastic pipette connected to a collection tube and vacuum line. A grand total of 98 oocytes were recovered, representing >90% of the number of follicles aspirated and an average of 32.6 oocytes/donor. Interestingly, 42/43 oocytes recovered from the 2 females that received hCG were showing expanded cumulus which has been shown to be an accurate sign of *in vivo* maturation and readiness for IVF, while from the female that was not injected with hCG all oocytes had compact cumulus and required *in vitro* maturation prior to further processing. IVM was performed in 50 µL drops of maturation medium under mineral oil, at 38.5°C in humidified atmosphere with 5% CO2 in air for 24 h. The maturation medium consisted of M199 supplemented with bLH (0.02 U/ml), bFSH (0.02 U/ml), 17β-estradiol (1 µg/ml), sodium pyruvate (0.2 mM), gentamycin (50 µg/ml) and 10% heat-inactivated fetal bovine serum. Unfortunately, we were unable to obtain semen to conduct IVF as originally planned, so we resorted into vitrifying the oocytes in order to store the oocytes until semen becomes available. The *in vivo* matured oocytes were vitrified shortly after collection while the immature oocytes were vitrified after completing 24h in the above-described IVM conditions. In all cases, prior to vitrifiation, the oocytes were completely stripped of the cumulus cells by pipetting in handling medium containing 0.1% hyaluronidase. Oocyte vitrification was conducted following the procedure by Vajta et al. Briefly, oocytes were sequentially passed through holding medium (HM, TCM199 supplemented with 20% FBS), HV1 medium (HM with 7.5% DMSO and 7.5% ethylene glycol, v/v), HV2 medium (HM with 1M sucrose plus 16% DMSO and 16% ethylene glycol, v/v) and finally placed in homemade cryotops (longitudinally hemi-sectioned 0.25 straws) prior to immersion in liquid nitrogen.

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A182 OPU-IVP and ET

Reduction in volume of Percoll gradients improves the quality and recovery of bovine sex-sorting for IVP

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Keywords: discontinuous percoll gradient, sperm selection for IVF, volumes.

The discontinuous Percoll gradient technique is one of the most used for animal sperm selection. However, in order to obtain a better use for conventional and/or sex-sorting semen pallets, besides reducing the costs of IVP and the IVF execution time, changes have been studied in the technic, such as different forces and time of centrifugation, number of Percoll gradients and volumes. This study aimed to evaluate the effect of different volumes of Percoll on the recovery membrane integrity and movement of bovine sexed sperm. Five replicates were conducted from a pool of sexed semen from two *Bos taurus taurus* bulls. The sperm selection was performed by discontinuous gradients (30, 60, 90%) Percoll (Folchini et al., Rev. Bras. Reprod. Anim., V.36, p.239-244, 2012), with volumes adjusted as treatments: Control: 300µL; Treatment 1 (T1): 100 µL and Treatment 2 (T2): 200µL for each gradient. At the first and second centrifugation a force of 2200 x g was used during 5' and 1', respectively. The samples were evaluated by a computerized system (SCA®, Sperm Class Analyzer, New Route, Barcelona, Spain) immediately after treatment concerning curvilinear velocity (VCL, µm/s) straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), beat cross-frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %), membrane integrity (staining with propidium iodide and 6-carboxyfluorescein diacetate) and sperm recovery rate obtained by the formula considering volumes and initial and final sperm concentrations (Machado et al., Theriogenology, v.71, p.1289-97, 2009). Data were analyzed by ANOVA ($p<0.05$). Reducing the volume of discontinuous Percoll gradients to 100 uL increased curvilinear velocity compared to the control and T2 (control: 34.4 um / s, T1: 70.2 um / s; T2: 43.9 um / s) and it was similar to T2 and higher than control as amplitude of lateral head displacement (Control: 1,3µm; T1: 3,5µm; T2: 1,8µm). About membrane integrity, it was observed that the Control (65.2%) and T2 (72.2%) were superior to T1 (55.8%). Evaluating the recovery rate, it was found that reducing the volume to 100 uL increased recovery rate (control: 28.3%; T1: 46.3%; T2: 28.8%) of sexed semen. These results demonstrate it is possible to obtain a higher recovery rate and selecting sperm motility with higher motility with the use of gradients 100 uL volumes.

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A183 OPU-IVP and ET

Embryonic survival and birth rate after the transfer of *in vitro* produced ovine embryos cryopreserved by slow freezing or vitrification with minimum volume methods

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Keywords: birth rate,cryopreservation, IVP ovine.

Recently has been reported in sheep IVP embryos at different stages of development a new vitrification methodologies with minimum volume method (Cryotop and Spatula MVD) (dos Santos Neto et al., 70, 17-22, 2015). The objective of this study was to evaluate the survival rate of embryos at day 30, birth and survival rate after the 1st week of life of *in vitro* produced sheep embryos cryopreserved by slow freezing with ethylene glycol and vitrified with Cryotop method or Spatula MVD method. A total of 240 embryos cryopreserved at morulae and blastocyst stage were transferred to recipient ewes divided into 3 experimental groups: Slow freezing (N = 68); Cryotop (N = 93); Spatula MVD (N = 79). The oocytes were matured, fertilized and cultured *in vitro* until day 6 of embryonic development following the protocol routinely used at the laboratory. On day 6 embryos were frozen with ethylene glycol or vitrified by both methods. The embryos were cryopreserved by slow freezing and vitrified with Cryotop method developed for humans and mice by Kuwayama et al., Theriogenology, 67, 73-80, (2007) or Spatula MVD method reported by our laboratory. The synchronization of the recipients was performed using a short term protocol and 6 days after heat detection (Day 0) cryopreserved IVP embryos were transferred. We evaluated embryo survival (number of embryos at 30 days / total embryos transferred) performed by trans-rectal ultrasonography (5MHz, Well-D, China), birth rate (born lambs / total lambs at 150 days), and survival rate the 1st week (live lambs the 1st week / total lambs born). Statistical analysis was performed by logistic regression with significance level P <0.05. The results demonstrated that embryos in vitro produced and vitrified with Cryotop method had a higher embryonic survival at day 30 when compared to those frozen with ethylene glycol as compared to vitrified with Spatula method MVD (Slow freezing 7.3% 5/68; Cryotop 38.7% 36/93; Spatula MVD 11.4%, 9/79; P <0.05), respectively. No differences were found in survival rates when the embryos were frozen with ethylene glycol or vitrified with Spatula method (Slow freezing 7.3% 5/68; Spatula MVD 11.4%, 9/79; P=NS). No differences were found in any of the groups assessed on birth rate (85.0% 34/40; P=NS) and survival of the first week (72.5% 29/40; P=NS). These results suggest that the achievement of acceptable survival rates at 30 days as well as the birth and survival of lambs the 1st week allow us to consider vitrification with Cryotop method as a possible tool to embryo transfer programs in sheep.

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A184 OPU-IVP and ET

Superovulation and non-surgical embryo recovery in Santa Inês ewes

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Keywords: sheep, superovulation, transcervical collection.

The objective of this study was to test the efficiency of two superovulatory protocolos and non-surgical embryo recovery in Santa Inês sheep. Sixteen pluriparous ewes were allocated to two experimental groups. G1 ewes (n=07) received intravaginal devices (60mg MAP; Progespon®, Syntex, Buenos Aires, Argentina) for six days, 37.5µg d-cloprostenol (Prolise®, ARSA S.R.L., Buenos Aires, Argentina) laterovulvar at sponge insertion and 24hs before its removal, 200IU eCG (Novormon 5000®, Syntex, Buenos Aires, Argentina) i.m. at device removal, and 200mg pFSH (Folltropin®, Bioniche, Canada) in eight decreasing doses (40, 40, 30, 30, 20, 20, 10, and 10mg) at 12h interval beginning two days before sponge removal. G2 (n=09) ewes received intravaginal devices for 15 days, 200IU eCG i.m. at sponge removal, and 200mg of pFSH i.m. in six decreasing doses (50, 50, 25, 25, 10, and 10 mg) at 12h interval beginning two days before sponge removal. Ewes were checked for estrous twice daily and natural mated by fertile rams while in estrus. In both groups, ewes received 0.025mg de GnRH (Gestran Plus®, ARSA S.R.L., Buenos Aires, Argentina) i.m. and 250IU hCG (Vetecor®, Hertape Calier, Barcelona, Espanha) i.m., 24 and 84h after estrous onset, respectively. All ewes received 37.5mg d-cloprostenol and 1mg estradiol benzoate (Estrogin®, São Paulo, Brasil) i.m. 16h plus 50IU oxytocin (Ocitocina forte ucb®, São Paulo, Brasil) i.v 20min before uterine flushing. Embryo collection was performed with ewes in standing position at day 7 estrous cycle by transcervical technique (Fonseca et al., Small Ruimin. Res., 111:96-99, 2013). Qualitative data were tested by qui-square test, and quantitative data were evaluated by ANOVA and t-test at 5% significance (SAEG®). The parameters were similar (P>0.05) for G1 and G2 ewes. Estrous response was 100%. The interval to estrus was 22.3 ± 4.5 and 16.0 ± 6.0h to G1 and G2 ewes, respectively. The percentage of ewes flushed was 85.7% (6/7) and 77.8% (7/9) G1 and G2 ewes, respectively. Overall flushing successful rate (liquid injected/liquid recovered) was 96.2%. The total (6.4 ± 7.9 and 7.4 ± 5.6) and viable (3.3 ± 4.7 and 2.7 ± 2.8) structures recovered was similar (P>0.05) to G1 and G2 ewes, respectively. Results of this study suggest that superovulated Santa Inês ewes can be subjected to non-surgical transcervical embryo recovery with satisfactory flushing efficiency and structure recovering.

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A185 OPU-IVP and ET

Nasal swab as pre test for herpesvirus seropositive oocyte donnors

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Keywords: BHV-1, nasal swab, oocytes.

Bovine herpesvirus is the infectious bovine rhinotracheitis disease (IBR) agent; this disease has a high prevalence in Brazil and is considered restrictive for international commercials trades of gametes and embryos. However, neutralization in microplates test is recommended by the OIE for diagnosis, but positive samples cannot differentiate vaccinated animals from non - vaccinated. The present study aimed to identify viral DNA in COCs and nasal mucosae from non-vaccinated and serologically BoHV positive cows as a method of pre selection of donors. Nine cows (n = 9) were separated into two experimental groups, six animals receive 0.1 mg / kg / bw / iv of dexamethasone (CORTVET, UCB saúde animal, Jaboticabal, Brasil) for five consecutive days (treated group – GT; n= 6), and in three animals (non treated group - GNT; n=3) were administered saline solution (0,05 mL/kg/ bw / iv) as the GT. The OPU and the nasal swab were performed on day 8 and 12 after the protocol began. The material was sent to PCR (Gasparini et al., Biologico, São Paulo, v.73, Suplemento 2, p.25-81, 2011). All nasal swabs and COCs samples were considered positive on PCR. The results show that PCR of nasal swab mighty have applicability as a tool for pre-selection of COCs donors and further studies are needed to validate this methodology for donors selection.

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A186 OPU-IVP and ET

Methyl beta cyclodextrin use as agent of sperm capacitacion on the *in vitro* production embryos buffaloes (preliminary results)

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Keywords: biopsy, IVF embryos, vitrification.

In Brazil, among animal reproduction biotechnologies, in vitro fertilization (IVF) is a tool that is being increasingly incorporated into the routine of beef and dairy cattle herds. One of the advantages of this technique is the use of sexed semen, which allows the producer to choose the sex of the embryo and therefore increase their productivity. However, there are limitations, due to the absence of sexed semen for some desired bulls. A solution to identify the sex of the embryo before implantation is embryonic biopsy. The objective of this work was to evaluate the rates of development of in vitro produced bovine embryos either intact or biopsied and transferred either fresh or cryopreserved. Embryos were produced on a commercial scale from aspirations of oocytes from Angus donors (FSL Angus; Itu, SP). On the sixth day of culture, embryos were submitted to biopsy of cells through the microaspiration technique, using two mechanical micromanipulators coupled to two borosilicate micropipettes (holding and biopsy). Approximately 10% of the embryo mass from the trophoblast was removed for sex identification by PCR technique. After micromanipulation, embryos were cultured individually and identified. After 8-12 hours, viable embryos were prepared for transfer (fresh) or subjected to vitrification. The transfer was performed into previously synchronized recipients. Pregnancy rates were analyzed by logistic regression with data considered significant with $P<0.05$. Day 60 conception rates were significantly lower ($P<0.02$) for biopsied, vitrified embryos (33.75%; 27/80) compared to biopsied, fresh (53.33%; 32/60), non-biopsied, fresh embryos (50.58%; 131/259) and non-biopsied, vitrified (54.78%; 63/115) embryos. The results of this preliminary survey show that the association between the two technologies, biopsy and vitrification, led to a significant loss of embryonic viability compared to these techniques individually. However, considering the possibility of obtaining a duly identified embryo bank according to the needs of the producer, and the flexibility that these techniques adds to IVF, further studies are necessary in order to preserve viability and increase conception rates of biopsied embryos after vitrification.

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A187 OPU-IVP and ET

Theophylline associated or not to heparin as a capacitation inducing agent to the *in vitro* production of bovine embryos

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Keywords: bulls, cleavage, fertilization.

The theophylline mechanism of action is similar to the caffeine mechanism. However theophylline is more efficient to increase the intracytoplasmic levels of adenosine 3',5'-cyclic monophosphate (cAMP), a nucleotide involved in the sperm capacitation process. The aim of this study was to evaluate theophylline as a capacitation inducer agent in replacement or in combination with heparin in the development of bovine embryos produced *in vitro*. The culture media used to produce the embryos were provided by Biodux® company (Campinas, SP, Brazil). Theophylline (T1633 - Sigma-Aldrich®) was added to the fertilization medium. The experiment was carried out with 4 Gir breed bulls and 3 treatments, in a total of 12 experimental groups. Each bull was evaluated according to the following treatments: Treatment 1 (T1): Heparin - 10mg / mL; Treatment 2 (T2): Theophylline - 5 mM; Treatment 3 (T3): Heparin (10mg / ml) + Theophylline (5mM). The cumulus-oocyte complexes (COCs) recovered from slaughtered cow ovaries were incubated in the maturation medium at 38.8ºC in atmosphere of 5% CO2 for 24 hours. In IVF step, the capacitating agents were added to the medium, composing the treatments T1, T2 and T3. The fertilization day was considered as day 0 (D0). The cleavage rate, embryo production and hatching were evaluated after 2, 7 and 10 days of fertilization, respectively. The data were submitted to variance analysis using generalized linear models. The averages were compared by Tukey Kramer test ($p < 0.05$). The embryo production rate was higher ($p < 0.05$) for the T1 (37.97 ± 13) relative to the T2 and T3 (10 ± 28.55, 27.60 ± 11.0, respectively). The same was observed in the hatching rate ($p < 0.05$) in the T1 (33.50 ± 14) compared to T2 and T3 (22.81 ± 11; 23.08 ± 10, respectively). Bull did not influence cleavage rates ($p > 0.05$) in the cleavage rates, produced embryos and hatching. Apparently, theophylline alone or associated with heparin did not induce a good sperm capacitation, which could be the reason of reduced fertilization rate. However, this fact was not evidenced by the cleavage rate, which was similar between treatments. This provides evidence that, although the rate of cleavage did not differ between treatments, not all of cleaved structures were competent to sustain the embryo development. These findings may be due the increased incidence of parthenogenesis or polyspermy and consequently blocking of embryonic development (Ramos et al., Brazilian Journal of Veterinary Medicine and Animal Science, V.52, 2000, online). Further investigations are necessary in order to explain the possible factors that led to theophylline alone or associated with heparin to reduce embryo production rates in in vitro fertilization.

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A188 OPU-IVP and ET

Methyl beta cyclodextrin use as agent of sperm capacitacion on the *in vitro* production embryos buffaloes (preliminary results)

A.X. Santos[1], V.C. Brito[1], A.A. Gonçalves[1], P.C.A. Ramos[1], S.T. Rolim Filho[2], H.F.L. Ribeiro[2], M.S. Miranda[1], S.S.D. Santos[1], A.R. Garcia[3], O.M. Ohashi[1]

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Keywords: buffalo embryo, cyclodextrin, sperm capacitation.

Cyclodextrins are cyclic oligosaccharides which have the ability of lipids such as cholesterol incorporation and can be used to alter the cell membrane cholesterol content (Visconti et al., 1999). Studies show their use in cholesterol removal from seminal membranes and in the induction of bovine sperm capacitation (Purdy et al, 2004, BiolReprod, 71, 522-527;Kato et al, 2010, Zygote, 19, 21. 30). Thus, the aim of this work is to evaluate the use of cyclodextrin as sperm capacitation agent, analyzing their influence on IVPE in buffalo. Buffalo ovaries were collected from slaughterhouse and the cumulus-oocyte complexes (COCs) were matured *in vitro* in TCM-199 medium supplemented with 10% FBS, FSH and LH, for 22 hours at 38.5 ° C in 5% CO2. COCs were fertilized in TALP - FERT medium supplemented with penicillamine, hypotaurine and epinephrine, modified according to the experimental groups: Negative Control - NC (without BSA, heparin or cyclodextrin), Positive Control - PC (with BSA and heparin as capacitation agent) and groups with different Methyl Beta Ciclodextrin (MBCD) concentrations (Sigma, St Louis, USA) (MBCD-0.5mM, MBCD-0.75mM and -MBCD-1.5mM) and incubated under the same conditions mentioned for IVM. 24 hours after fertilization, the zygotes were placed on SOF medium drops supplemented with BSA (6 mg /mL), 10% FBS, aminoacid, pyruvate, gentamicin and antioxidant. The cleavage and blastocyst rates were evaluated on the 2nd, 6th and 7th cultivation day, respectively, and the results were analyzed by ANOVA and Tukey post-test, adopting the significance level of 5%. The cleavage rate there was no significant difference between PC and MBCD-0.5 mM (0 vs 39.23 ± 4.06 and 32.15 ± 17.25), but NC differed MBCD-0.75mM and MBCD-1.5mM (0 vs 46.94 ± 17.00 and 46.45 ± 17.70, respectively), but it had no significant difference between the groups with MBCD. In the production of blastocysts at 6th day of culture, the PC groups (22.25 ± 5.75), MBCD- 0.75mM (18.18 ± 8.27) and MBCD-1, 5mM (17.77 ± 6.74) did not differ significantly ($p>0.05$), however they differ from NC groups and MBCD 0.5mM (0 vs. 10.74 ± 6.24, respectively) ($p<0.05$). And at 7th day of culture PC groups (33.97 ± 3.38) and MBCD- 1.5mM (25.90 ± 10.68) are not different, but differed from NC (0), MBCD-5mM (11.36 ± 10.54) and MBCD-0.75mM (18.18 ± 8.27). Thus, more repetitions are needed in order to confirm that the use of buffaloes MBCD in concentration of 1.5mM shows similar results to the positive control, thus demonstrating that MBCD can be used as a heparin substitute.

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A189 OPU-IVP and ET

Viability of transcervical embryo transfer in goats

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Keywords: cervical via, goat, non-surgical embryo transfer.

Embryo transfer consists in the deposition of the embryo in the recipient uterus. The results of recipient's fertility vary widely depending on the origin of these embryos: fresh or cryopreserved and produced in vivo or in vitro. Typically, pregnancy rates ranging from 40 to 80% are reported. The objective of this study was to test the feasibility of non-surgical embryo transfer of goat fresh embryos. Pluriparous goats (n=28) received intravaginal sponges containing 60 mg medroxy acetate progesterone (MAP; ProgesponÒ, Syntex, Buenos Aires, Argentina) for six days, 30mg d-cloprostenol (ProliseÒ, ARSA S.R.L., Buenos Aires, Argentina) latero-vulvar and 200 IU eCG (NovormonÒ 5000; Syntex, Buenos Aires, Argentina) i.m., both at 24 h before sponge removal. After sponge removal, females were teased individually every 12 h to identify the onset and end of estrus. All animals were evaluated by transrectal ultrasound one day before transfer for identification of corpora lutea. At the seventh day of estrous cycle, the females received embryos by transcervical via, through the use of Collin speculum, Allis forceps and urethral catheter (Arq. Bras. Med. Vet. Zoo., 66(2):613-616, 2014). As the technique is minimally invasive, similar to routine procedures as Artificial Insemination, anesthesia was not used. The following end points were evaluated: type (morula/blastocyst) and classification (grade I, II and III) of embryos, the number of embryos transferred per recipient, number of corpora lutea presented per recipient and uterine horn receiving the embryo (right/left). For data analysis, logistic regression models (univaried and multivariate) were performed using the Epi Info version 3.5.3 software. All synchronized females presented estrus. The pregnancy rate was superior ($P<0.05$) to blastocysts (45.5%; 5/11) when compared with morulae (16.0%; 4/25) and for Grade 1 embryos (55.6%; 5/9) compared to Grade 2 (23.1; 3/13) or Grade 3 (7.1%; 1/14). There was no effect ($P>0.05$) in the number of embryos transferred per recipient or uterine horn to which the embryos were transferred on the pregnancy rate. Recipients presented from one to three corpora lutea per ovary and the number of corpora lutea did not affect the pregnancy rate ($P>0.05$). At parturition, 14 kids were born representing 38.9% of birth (14/36). The results of this study suggest that embryo transfer may be successfully performed by the non-surgical via in goats, representing an alternative to the surgical traditional procedure.

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A190E OPU-IVP and ET

Fertility effects of performing ovum pick up at young age

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Keywords: fertility, flushing AI index, IVP, OPU.

To shorten the generation interval, and to increase the genetic progress, CRV started to perform Ovum Pick Up (OPU) at 9 months instead of 12 months of age (Reproduction Fertility and Development 12/2014; 27;:209). We demonstrated that animals that had their (first) estrus before the first OPU produced significantly more embryos than animals that did not show estrus before OPU. It is however not known however what the effect is of performing OPU on such young animals on their fertility (i.e. flushing results and AI index).The aim of this study is to check the fertility of animals that have been used for OPU at young age. To investigate this we compared the flushing results and AI index of these animals. Embryos were produced by OPU-IVP (once every week during a period of 4-9 weeks), followed by flushing (two times) and insemination (AI) to make the animals pregnant. We used 3 groups of animals, (1) 12 young animals (9-10 months) that had their first estrus before the OPU, (2) 24 young animals that did not had their first estrus before the OPU and a (3) control group of 16 older (12-14 month) animals. The flushing results from young animals that had their first estrus before OPU (Group 1) were comparable with those of the control group (both 6.5 embryos per flush). However, flushing results from young animals that did not had their first estrus before OPU (group 2) were clearly lower and had only 4.1 embryo per flush.Interestingly, the insemination results (AI index) showed the same tendency, e.g. animals that had their first estrus before needed 2.1 semen straws to get pregnant, while animals that did not had their estrus before OPU needed 2.6 straws. It is therefore concluded that in young animals that showed estrus before the first OPU no difference in flushing results and AI index fertility results later in life were observed as compared to older animals. However, when no estrus was observed before the fist OPU, fertility results were lower. It is not known if this is due to the OPU at young age or that these are less fertile animals having estrus at a later stage.

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A191E OPU-IVP and ET

The use of neutral red as a viability indicator hampers *in vitro* development of semi-nude bovine oocytes to the blastocyst stage

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Keywords: Neutral Red, oocyte viability, semi-nude oocytes.

Women suffering from premature ovarian failure due to cancer treatment can appeal to oocyte vitrification to preserve their fertility. An important factor to increase the effectiveness of the procedure is viability assessment of the cryopreserved oocytes after warming. To date, survival is predominantly assessed on the basis of morphological criteria by conventional light microscopy, a subjective assessment method that depends largely on the expertise of the observer. Therefore, there is a great need for an objective method to assess viability in a fast and non-invasive way. Oocytes can be cryopreserved at the immature or mature stage. After choosing to use mature oocytes, based on literature, this experiment examined whether the relative non-toxic stain Neutral Red (NR) can be used as an oocyte viability marker without affecting subsequent development to blastocysts. NR is taken up by lysosomes of metabolically active cells. Briefly, immature cumulus-oocyte-complexes (COCs) were subjected to routine *in vitro* maturation (IVM) for 21 or 24 hours, whereupon the 270 mature COCs were divided into 3 groups (2 replicates). A control group with an intact cumulus oophorus (24h IVM; LAB CTRL) and 2 groups of COCs with only the corona radiata (21h IVM), the semi-nude (SN) and Neutral Red group (NR) respectively. In view of future vitrification and IVF, cumulus cells were partially removed (semi-nude) by pipetting to facilitate oocyte handling and future cryoprotectant penetration. Following 30 minutes incubation with 15µg NR/ml maturation medium and a subsequent 1h washout period (NR group), all 3 groups were subjected to routine IVP (cultured under oil for 8 days). Cleavage and blastocyst rate were observed at respectively 2 and 8 days post-insemination. Developmental competence data were analyzed using a binary logistic regression including treatment as fixed factor and replicate as random factor (IBM SPSS version 22). Although there is a significant difference in cleavage (75 vs 55.8%) and blastocyst (36 vs 20.9%) ratio between the LAB CTRL and SN group, our results demonstrate that semi-nude oocytes still have an acceptable fertilization rate that can definitely be improved. However, oocytes from the NR-group significantly failed to cleave (42.9%) and develop to the blastocyst stage (2.4%) as compared to the CTRL and SN group. In conclusion, Neutral Red clearly affects cleavage and blastocyst formation of semi-nude oocytes in the above used conditions and therefore is not suitable for semi-nude oocyte viability assessment.

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A192E OPU-IVP and ET

Influence of caffeine supplementation prior to *in vitro* maturation on bovine oocyte developmental capacity

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Keywords: caffeine supplementation, cattle, *in vitro* maturation, oocyte.

Although, *in vitro* oocyte maturation (IVM) is common practice in the cattle industry, it is known that the mechanisms involved in meiotic resumption begin in a non-physiological way. The cyclic AMP pathway plays an important role in resumption of meiosis. When cumulus-oocyte-complexes (COC) are mechanically released from the follicle to perform IVM, cAMP levels in immature COC rapidly decrease, which in turn triggers meiosis continuation. It has been proposed that modulation of cyclic AMP prior to IVM can increase bovine blastocyst rates *in vitro*. Caffeine is a non-specific competitive phosphodiesterases (PDE) inhibitor and can inhibit meiotic resumption of oocytes due to maintenance of cAMP levels. It has been reported that gamete treatment with caffeine can increase developmental potential. The present study evaluated the influence of pre-IVM culture in the presence of different concentrations of caffeine on meiotic progress, developmental rates and blastocyst cell numbers. Bovine ovaries were collected from a local abattoir. A total of 4378 cumulus-oocyte-complexes were obtained by slicing. Four different concentrations of caffeine (Merk, Darmstadt, Germany) were used during slicing, searching and 2h pre-IVM culture: 1, 5, 10, 20 mM. A control group, using 2h pre-IVM without caffeine (0mM) and a standard control were also included. After pre-IVM, oocytes were washed and cultured for 24h *in vitro* without caffeine. Following maturation, oocytes were fertilized *in vitro* for 19h and zygotes were cultured *in vitro* for eight days to assess embryo development. Some oocytes were fixed in 2% glutaraldehyde at 9, 20 and 24 h after IVM. Hoechst staining was performed to evaluate nuclear status. Cleavage and blastocyst formation rates were evaluated. Expanded blastocysts from all treatments were submitted to differential staining. One-way ANOVA from R software was implemented to evaluate differences in progression through meiosis, cleavage and blastocysts rates and blastocyst cell numbers. Caffeine maintained the meiotic arrest after 9h IVM in a concentration dependent manner (GV: 100 ± 0.0%, 61.3 ± 21.3%, 40.7 ± 5.4 %, 36.2 ± 11.4% 11.9 ± 6.3%, 28.5 ± 10.0% for 20, 10, 5, 1, 0 mM and standard, $p<0.05$, mean ± SEM). Cleavage (57.7 ± 4.9%, 56.5 ± 3.8%, 62.7 ± 3.2%, 52.5 ± 5.1%, 54.4 ± 6.0%, 60.3 ± 2.3% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) and blastocyst rates (26.2 ± 3.0%, 14.9 ± 2.8%, 22.4 ± 3.8%, 23.7 ± 2.1%, 21.4 ± 4.1%, 26.6 ± 2.4% for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean ± SEM) and number of cells (ICM: 46.0 ± 4.1, 43,2 ± 3.7, 61.4 ± 7.8, 53.0 ± 6.5, 49.4 ± 5.6, 50.0 ± 4.4; TE:111.6 ± 13.6, 115.4 ± 7.8, 106.4 ± 3.5, 102.6 ± 8.3, 118.4 ± 14.6, 119.6 ± 11.7 for 0, 1, 5, 10, 20 mM and standard, $p>0.05$, mean±SEM) did not differ significantly among *in vitro* treatments. Although caffeine supplementation prior to IVM delayed resumption of meiosis, it did not affect subsequent embryo development and quality.

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A193E OPU-IVP and ET

Temporal pattern of steroid hormone concentrations during *in vitro* maturation of bovine oocytes

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Keywords: cattle, *in vitro* maturation, steroid hormones.

Present in vitro maturation (IVM) systems do not completely mimic the in vivo situation resulting in oocytes of reduced quality. Steroid hormones are regulators in the fine-tuned mechanism of follicular and oocyte maturation and development. During final maturation a switch from estradiol dominance to progesterone dominance within the follicle is well-described. This change is accompanied by the resumption of meiosis and results in the maturation of the oocyte. It also suggests the important role of these hormones in this process. Aim of the study was to determine the temporal pattern of steroid hormone concentrations in the IVM medium of bovine cumulus-oocyte-complexes (COC) supplemented with different gonadotropin concentrations. COC were obtained from abattoir-derived ovaries and were matured in medium TCM 199 (Tissue Culture Medium 199) supplemented with three different compounds of gonadotropins employing a standard protocol. The three combinations of gonadotropins were: 1. equine (eCG) and human chorionic gonadotropin (hCG), 2./3. follicle-stimulating hormone (FSH) and luteinizing hormone (LH), each in two different concentrations 0.05 IU or 0.01 IU, and 4. without any supplementation of gonadotropins. Groups of 30 COC were matured for 24 hours at 39°C and 5% CO^2 without oil overlay. 17β-estradiol (E2) und progesterone (P4) were measured in maturation medium before use (0h, control) and after specific time points of IVM via radioimmunoassay (RIA). So far, the following results could be obtained. *Treatment 1: TCM with eCG and hCG:* P4 and E2 could not be detected in the control medium (0h). During IVM, P4 concentrations increased in the medium (4h: 3.3 ± 1.0 ng/ml; 8h: 6.2 ± 3.3 ng/ml; 12h: 6.5 ± 2.0 ng/ml; 16h: 6.8 ± 1.1 ng/ml; 20h: 7.3 ± 1.8 ng/ml; 24h: 10.4 ± 1.6 ng/ml), whereas the E2 concentrations stayed similar (4h: 52.8 ± 12.1 pg/ml; 8h: 54.6 ± 7.9 pg/ml; 12h: 63.8 ± 15.2 pg/ml; 16h: 54.2 ± 16.3 pg/ml; 20h: 77.1 ± 40.1 pg/ml; 24h: 74.7 ± 32.4 pg/ml). Treatment 2/3: *TCM with FSH and LH:* Supplementation of 0.05 IU each, E2 concentrations stayed at the same level as with eCG and hCG (E2 0h: 6.2 ± 5.7 pg/ml, washing medium: 26.0 ± 10.8 pg/ml, after 24h: 59.7 ± 20.1 pg/ml). With the supplementation of 0.01 IU each, P4 and E2 concentration also stayed at the same level as with eCG and hCG (E2 0h: 3.3 ± 3.2 pg/ml, washing medium: 19.6 ± 4.2 pg/ml, after 24h: 58.4 ± 24.1 pg/ml, P4 0h: ≤0.25 ng/ml, washing medium: 0.3 ± 0.1 ng/ml, after 24h: 17.3 ± 3.5 ng/ml). *Treatment 4:* After 24 h of IVM *without gonadotropins* the following hormone concentration could be detected: E2: 129.4 ± 88.8 pg/ml and P4: 6.7 ± 0.8 ng/ml. During IVM, the temporal pattern of E2 and P4 did not correspond with the pattern during final maturation in vivo. This underlines that present conditions of IVM do not reflect the in vivo situation and require further optimisation.

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A194E OPU-IVP and ET

Time-lapse analysis of early cleavage in bovine embryos produced in serum-free medium

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Keywords: cattle, embryo cleavage, IVP, time-lapse cinematography, viability.

Two decades ago, early cleaving embryos were considered as developmentally more competent than slow cleaving embryos. But this theory has been challenged, since moderately developing embryos have decreased chromosomal abnormalities, normal *H19* and *Snrpn* imprint maintenance and potentially higher pregnancy rates. We want to analyze the incidence of chromosomal instability (CIN) in bovine cleavage stage embryos and relate this with developmental kinetics. Hence, we need a culture system allowing individual identification and selection of cleavage stage embryos for single cell analysis (SCA). In this preliminary study, we used time-lapse cinematography (TLC) as a non-invasive tool to describe kinetics and to use timing of early cleavages as a parameter predictive of blastocyst development. Bovine embryos were produced from immature oocytes derived from slaughtered cattle. Oocytes were matured in 500 µL TCM199 supplemented with 20 ng/mL epidermal growth factor (EGF). After in vitro fertilization with frozen-thawed bull semen, 9 presumed zygotes (7 replicates) were cultured in a WOW dish in 30 µL Synthetic Oviduct Fluid (SOF) supplemented with 0.4% BSA, 5 µg/mL insulin, 5 µg/mL transferrin and 5 ng/mL selenium (ITS), covered with mineral oil. In total, 63 zygotes were observed with TLC (Primo Vision®, VitroLife, Göteborg, Sweden), and images were taken every 15 min for up to 90 hours post insemination (hpi). At 192 hpi, blastocyst formation was set as endpoint. Timing of the first (t_1; cleavage into 2-cell stage) and second mitosis (t_2; cleavage into 4-cell stage) and the interval time between those two parameters were analyzed ($t_{\Delta 1\text{-}2}$). The median observation of each parameter was set as a threshold value (t_1 29.00h; t_2 38.83h; $t_{\Delta 12}$ 10.87h). All data were analyzed using a binary logistic regression model. Significantly more embryos reached the blastocyst stage when they cleaved before 29.00h into 2-cell stage or before 38.83h into 4-cell stage (48.3% and 51.2%, respectively), compared to embryos with a later first or second mitosis (16.1% and 18.9%, respectively) ($P<0.05$). Furthermore, when the interval between the first and second mitosis ($t_{\Delta 12}$) was shorter than 10.87h more embryos reached the blastocyst stage (42.3%), compared to a longer interval $t_{\Delta 12}$ (21.1%) ($P<0.01$). This indicates that timing of early cleavage is predictive for further developmental potential, which is confirming earlier studies (Van Soom *et al*., Theriogenology, 38:905-919, 1992; Grisart *et al*., J Reprod Fertil, 101:257-264, 1994). It is however the first time embryos have been cultured in WOW-dishes in serum-free medium and monitored using TLC. WOW dishes offer the advantage of small group culture with individual embryo follow-up, which allows specific embryo selection at any time of the development. Next, we want to identify CIN in embryos with particular cleavage patterns using TLC with SCA and eventually transfer embryos with high and low predicted viability.

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A195E OPU-IVP and ET

3D visualization of bovine oocyte *in vitro* maturation by confocal laser scanning microscopy

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Keywords: bovine, fluorescence microscopy, oocyte maturation.

Regular nuclear and cytoplasmic oocyte maturation is a prerequisite for normal fertilization and embryo development. Improvement of *in vitro* maturation systems is a central issue in veterinary and human reproductive medicine. Thereby, better microscopic visualization of cellular processes and structures is essential for further extending our rudimentary knowledge and understanding of mammalian oocyte maturation. We used three-dimensional multicolor fluorescence microscopy to investigate critical steps of meiotic maturation *in vitro*. The primary aims of this study were to simultaneously gain information on the meiotic spindle apparatus, on the kinetics of meiotic progression, on the dynamic changes of the cytoskeleton and on the meiotic failures and aberrations. In cattle, the cumulus oophorus is considered to play an essential role for normal oocyte maturation. This makes direct microscopic live cell imaging of the oocyte rather difficult. Thus, cumulus-enclosed grade I and II oocytes were collected from slaughterhouse ovaries and allowed to mature for variable times from 0 to 28 hours *in vitro*. The oocytes were denuded and then fixed with formaldehyde in a microtubule-stabilizing buffer in such a way that the three-dimensional cell architecture was maintained, and were stained for DNA, microtubules and f-actin microfilaments. In addition, serine 10-phosphorylated histone H3 was used as a marker for chromosome condensation and the spindle midbody. For three-dimensional imaging of the oocytes *in toto*, confocal serial sections were captured at 1 µm distance using a 40x objective (NA = 1.3). For imaging details we used a high spatial sampling density (pixel size 50 x 50 nm, z-step size 200 nm) and image restoration by maximum likelihood estimation (MLE) deconvolution. A collection of more than 500 confocal image stacks gives a clearer and more detailed view of completion of meiosis I and progression to metaphase II. Qualitative and quantitative data analyses provide a basis for studies on molecular mechanisms e.g. on the dynamic localization and function of potential key proteins. Important is the detection of anomalies of meiosis I that result in irregular genomic configurations in the zygote: Main findings were (i) the failure of first polar body extrusion as a consequence of incorrect positioning or orientation of the meiotic spindle and (ii) lagging chromosomes, chromatin bridges and incomplete polar body cytokinesis due to irregular spindle formation, chromosome congression and segregation. 3D fluorescence microscopy allows to exactly determine the stage of oocyte meiosis and to diagnose fatal aberrations of meiotic maturation. Thus, high speed imaging systems could be used to test and to improve oocyte isolation methods and in vitro maturation systems.

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A196E OPU-IVP and ET

Concentration of procaine and exposure time influence *in vitro* fertilization rate in the equine

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Keywords: horse embryo, *in vitro* fertilization, procain exposure.

Most wild equids are currently endangered or threatened, as mentioned in the Red List of the International Union for the Conservation of Nature and several domestic horse breeds are at risk of extinction. Genome resource banking requires cryoconservation of semen, oocytes and/or embryos. Embryo production in equids is limited *in vivo*, since routine induction of multiple ovulation is still ineffective. Embryo production *in vitro* allows the production of several embryos per cycle that could easily be frozen owing to their small size. Intracytoplasmic Sperm Injection (ICSI) has been widely adopted to generate horse embryos *in vitro*, however ICSI is time-consuming and requires expensive equipment and expertise in micromanipulation. We have established an efficient *in vitro* fertilization (IVF) technique in the equine (Ambruosi et al., 2013 Reproduction, 146: 119-133) but IVF zygotes have a low developmental competence. Incubation of gametes with procaine, necessary for induction of sperm hyperactivation, may have a deleterious effect on embryos quality. Our objective was to increase the developmental competence of the IVF zygotes by decreasing procaine concentration or exposure time. Immature cumulus-oocyte complexes were collected from slaughtered mares in a local slaughterhouse, cultured for 26 hours in an *in vitro* maturation medium and pre-incubated for 30 minutes in oviductal fluid collected from slaughtered females. Fresh sperm was collected, diluted to $10x10^6$ spermatozoa/ml, incubated for 5 hours in a capacitating medium and diluted to $1x10^6$ spermatozoa/ml. Spermatozoa were then added procaine (1mM or 5mM) and co-incubated with oocytes for 2, 4 or 18 hours. Zygotes were cultured in DMEM-F12 for 48 hours post-IVF, fixed and analyzed. In experiment 1, spermatozoa were added 5mM procaine and co-incubated with oocytes for 2 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was higher for 18 hours co-incubation (62%, 13/21) than for 2 hours (0%, 0/22) (Chi2 test $p<0.05$). In experiment 2, spermatozoa were added 5mM procaine and co-incubated with oocytes for 4 hours *vs* 18 hours. The percentage of zygotes 48 hours post IVF was similar for 18 hours (44%, 7/16) and 4 hours co-incubation (32%, 6/19) (Chi2 test $p>0.05$). In experiment 3, spermatozoa were added 5mM *vs* 1mM procaine and co-incubated with oocytes for 18 hours. The percentage of zygotes 48 hours post IVF was higher for 5mM procaine (48%, 13/27) than for 1mM (19%, 5/26) (Chi2 test $p<0.05$). In the 3 experiments, zygotes contained at least 2 highly decondensed pronuclei, pronuclei decondensation being the first step of embryo development. We also observed 2 cleaved embryonic structures in the group 5mM during 18 hours, but the quality of these embryos was poor. In conclusion, decreasing procaine concentration or exposure time influence IVF rate and doesn't improve equine embryo quality.

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A197E OPU-IVP and ET

Reproductive success in interbred ewes after fresh embryo transfer

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Keywords: MOET, pregnancy, Romanov sheep, Turkish native sheep.

Estrous response and ovulation rate of Turkish Native Sheep (N; n=27) and the prolific Romanov crossbred breed (NR; n=22) for the use as recipients in embryo transfer programs were evaluated in the anoestrus season (April). Estrus of recipient ewes was synchronized with vaginal sponges containing 30 mg FGA for 12 days and ewes received an i.m. injection of 400 I.U. PMSG at sponge removal. Estrus and ovulation rates, time to onset of estrus and site of the ovulation were determined. A total number of 3 Romanov breed donors ewes were superovulated using FSH-p with 200 mg NIH-FSH-P1 (total of 20 ml) (Folltropin-V; Vetrepharm, Canada) applied in 8 decreasing doses of 1.5, 1.5, 1.5, 1.25, 1.25, 1, 1, 1 ml i.m. at 12 h intervals, starting 60 h before sponge withdrawal. Donors received 1ml Estrumate and 100 I.U. PSMG 36h prior to sponge removal and finally an additional 200 I.U. PMSG was injected at sponge removal. Donors were mated with their own breed of rams. Ewes were observed for estrus (d 0) and were surgically flushed by laparotomy 6 or 7 d later to recover embryos. The number of ovulations and transferable embryos were 18.7 and 14.3, respectively. Embryos with Grade I, II and III with the stage of morula to expanded blastocysts were used in twin fresh transfer. The success rate of the synthetic progestagen treatment to establish an estrus out of season (April) was found to be 59.3% and 52.4% for N and NR, respectively. Time between the removal of the sponges and the onset of estrus was similar between the two breeds of recipient ewes (N: 53.06±0.95h and NR: 52.27±1.07h). For the recipient ewes at ET the ovulation rates were found significantly higher ($P<0.05$) in N ewes (1.0±0.00) than NR ewes (0.72±0.14); the ovulation site was mainly located on the right ovary in NR ewes (87.5%) compared to N ewes (42.9%). We transferred embryos as pairs to save number of recipients as advised by Gimenez-Diaz (2012) who indicated that pregnancy success for number of embryos transferred (single versus twin) was similar. Recipient ewes with fertile estrus (estrus accompanied with ovulation) received similar stages of embryos fallowing the laparoscopic measurements (location, number and quality score) of CL in recipients. Pregnancy and embryo survival rates were similar in N (64.3% and 77.8%) and NR (75.0% and 75.0%) ewes. The sex ratio of twin transferred embryos was higher in N (75% male) than those observed in NR (22.7% male) ewes. These preliminary results show that, Romanov crossbreed recipients with a lower ovulation rate and ovulation occurred mostly in right ovary had more overall MOET success (66% vs 57%; no of lambs born/no of embryo transferred) and were found more favourable with more female lambs from fresh embryo transfer compared to Turkish Native Sheep.

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A198E OPU-IVP and ET

Estradiol route and non-surgical embryo recovery in synchronized Santa Inês ewes

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Keywords: embryo recovery, estradiol benzoate, Santa Inês ewes.

Animal surgery procedures are being progressively restricted worldwide in the context of embryo transfer. In small ruminants, the needs for development of alternative and efficient non-surgical techniques for embryo transfer have been emphasized. Non-surgical embryo recovery is well consolidated in Brazil in cattle and goats, while in sheep it remains a challenge. The aim of this study was to check the efficiency of different ways of estradiol benzoate administration on cervix dilation and embryo recovery in synchronized Santa Inês ewes. A total of 23 pluriparous ewes were subjected to two doses of 37.5 μg d-cloprostenol by intravulvo-submucosal way seven days apart. After the second cloprostenol administration, ewes were checked for estrus at 12 h interval and mated with fertile rams during estrus. After mating, ewes were allocated according to estrous response into two treatment groups for embryo recovery seven days after estrous onset. In T1 (n=11), ewes received 37.5 μg d-cloprostenol and 1 mg estradiol benzoate 16 h before embryo recovery, plus 50 IU oxytocin i.v. 20 min before embryo recovery. In T2 (n=10), ewes received the same protocol as T1, but the way of estradiol administration was intravaginal. All ewes received 2 ml of lidocaine 2% without vasoconstrictor for epidural and 2 ml of lidocaine for contact cervical anesthesia plus acepromazine 1% (1 ml/kg live weight) before cervical passage as previously described in goats (Fonseca et. al.; Small Rumin. Res., 111:96-99, 2013). Qualitative and quantitative data were analyzed by chi-square test and ANOVA respectively with 5% significance. Estrous response after the second cloprostenol administration was 91.3% (21/23). There were no differences (P>0.05) in any parameter evaluated for T1 and T2: successful uterine flushing (90.9% and 80.0%), duration time of embryo recovery (20.3 ± 8.0 and 26.2 ± 5.3min), flushing recovery rate (PBS injected/PBS recovered; 90.1 and 90.5%), average structures recovered (1.0 ± 0.4, 20% viable and 1.4 ± 0.6, 33% viable). Considering that Santa Inês sheep have up to 1.3 lambs we can conclude that it is possible to perform efficient non-surgical embryo recovery in non-superovulated synchronized Santa Inês ewes, regardless the way of estradiol administration.

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A199E OPU-IVP and ET

Effect of Thymosin on *in vitro* fertilization and developmental competence and quality of pig embryos

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Keywords: IVF, pig embryo quality, thymosin.

Thymosin (TH) is biological active polypeptide released by thym. It plays a vital role in the repair and regeneration of injured cells and tissue. It protects cells from damage and blocks apoptosis (Goldstein et al. 2012, Expert Opin Biol Ther, 12(1), 37-51). In our recent study we demonstrated that supplementation of maturation medium for pig oocytes with synthetic TH increased the number of matured oocytes with lower morphological quality. The aim of the present study was to investigate the effect of supplementation of maturation medium for oocytes with TH on *in vitro* fertilization and developmental competence and quality of pig embryos. Cumulus-oocyte complexes (COCs) were obtained by aspiration from antral follicles of ovaries collected from slaughtered gilts. COCs were selected based on their cytoplasm morphology and cumulus cells appearance and cultured in modified TCM-199 medium supplemented with 0.5 mg/ml of synthetic TH (LipoPharm.pl) (experimental group) or without TH (control group) for 42 h at 39^{o}C and in a humidified atmosphere containing 5% CO_2 in air. After maturation, oocytes were assessed and *in vitro* fertilized (IVF). Semen for IVF was incubated in modified capacitation medium-M-199 for 1 h. Sperm fraction was introduced to the droplets containing oocytes and next gametes were coincubated for 4 h in modified TCM-199 medium. Presumptive zygotes were cultured *in vitro* for 144 h in NCSU-23 medium under the conditions stated above. Embryo quality criteria were cleavage, morula and blastocyst rates, total cell number per blastocyst and degree of apoptosis assessed by TUNEL. The results were analyzed statistically with Chi-square test. Treatment of oocytes with TH during culture slightly increased the ratio of matured oocytes (95/103, 92.3%) compared to the control group (134/150, 89.3%; no significant differences) cultured without TH. After IVF cleavage, and development to the morula and blastocyst stage, based on number of cleaved embryos, were not different between experimental (29.5, 71.4 and 32.1%, respectively) and control (25.4, 50.0 and 29.4%, respectively) group. The mean number of cells per blastocyst in experimental and control group was comparable (40.4 and 39.9; respectively). The mean number of apoptotic nuclei and apoptotic index was 0.67 and 1.66 in the experimental group and was significantly lower ($P<0.05$) than in the control group i.e. 1.66 and 4.35. In conclusion, the culture oocytes in a medium with TH supplementation had a positive effect on quality pig IVF blastocyst since they had a significant lower incidence of apoptosis. However, further studies are required to determine the competence of porcine blastocyst recovered from oocytes matured with TH for *in vivo* development.

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A200E OPU-IVP and ET

A retrospective study of *in vitro* embryo production from high genetic merit cows using unsorted or X-sorted sperm in a commercial program

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Keywords: breeding program, cattle, IVP, semen sexing.

X-sorted sperm can be used in embryo transfer programs to produce female progeny (Kaimio et al., Theriogenology 80, 950-954, 2013). However, X-sorted sperm is generally used in heifers as cow insemination results in lower numbers of transferable embryos (Hayakawa et al., Theriogenology 71, 68-73, 2009). We hypothesized that breeding programs based on IVP with X-sorted sperm may be a promising alternative. The aim of this study was to compare *in vitro* embryo production in cows using X-sorted or unsorted semen under commercial conditions performed at the Biotechnology MIDATEST Station located in Denguin, South West, France. Three to fifteen years old Holstein cows (n=26) and 16-22 months old heifers (controls: n=17) were used in an OPU-IVP program. Donor cows were stimulated with decreasing pFSH doses (Stimufol; Reprobiol, Liège, Belgium) twice daily during 3 days, (total dose: 350 µg for cows and 250 µg for heifers). Cumulus oocyte complexes (COCs) were collected by OPU 12 to 24 h after the last FSH injection and *in vitro* matured using a standard IVM protocol. Oocytes were fertilized with frozen–thawed unsorted (cows and heifers) or X-sorted (cows) sperm in modified Tyrode's bicarbonate buffered solution medium (fert-TALP) using different non pre-tested bulls (n=55). Presumptive zygotes were cultured in SOF medium (Minitub, Tiefenbach, Germany) plus 1 % cow serum up to Day 7 at 38.5 °C in 5% CO2, 5% O2 and 90% N2 atmosphere with maximum humidity. OPU/IVP was repeated one to 13 times (2.5 ± 2.6) for each donor cow or heifer. Grade 1 blastocysts and expanded blastocysts according to IETS classification were recorded on days 6.5 and 7. Viable embryos were frozen or transferred fresh. Embryo production was analyzed with ANOVA and blastocyst yield by Chi-Square. From 18 OPU sessions in heifers, a total of 168 COCs (9.3 ± 4.7 per session) were processed for *in vitro* maturation, and 5.4 ± 3.9 Grade 1 (G1) embryos were produced per session. In cows 42 sessions were performed with unsorted semen and 44 with X-sorted semen, 13.1 ± 9.6 and 8.9 ± 4.9 oocytes ($p<0.05$) were collected; 7.7 ± 5.5 and 4.1 ± 2.9 G1 embryos were produced, respectively ($p<0.05$). The mean embryo development rate (total number of G1 embryos / number of oocytes entering maturation process) was 59.1% (unsorted semen) and 46.3% (X-sorted semen; $p<0.05$). Although the number of collected oocytes was different, there were no differences in presumptive female embryos produced per session assuming a sex ratio of 90% (3.7 embryos per session) with sorted semen and a sex ratio of 50% (3.9 embryos per session) when using unsorted semen. In conclusion, our work confirmed the efficacy of OPU-IVP techniques to produce grade 1 embryos using X-sorted in high genetic merit cows. Furthermore this technique allows to get female calves based upon a lower number of recipients.

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A201E OPU-IVP and ET

Presence of L-carnitine during maturation improves efficiency of fertilization in porcine oocytes

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Keywords: *in vitro* fertilization, *in vitro* maturation, L-carnitine, pig embryo.

The lipid-rich pig oocytes might be an excellent model to understand the role of fatty acid metabolism during oocyte maturation, their subsequent monospermic fertilization and preimplantation embryo development. Recently, it was described that L-carnitine stimulates mitochondrial oxidation of fatty acids and increases energy supply of mammalian oocytes. The aim of the study was to characterize the effect of L-carnitine during maturation on the efficiency of fertilization of porcine oocytes with different meiotic competence. Cyclic sows, checked for the ovarian cycle status, were used as oocyte donors. Meiotically more competent (MMC) and meiotically less competent (MLC) oocytes were isolated either from medium (6–9 mm) or small follicles (<5 mm). They were matured separately in IVM medium supplemented with 0, 4 and 10 mM L-carnitine (Sigma-Aldrich Co., Prague, Czech Republic) and fertilized by frozen-thawed spermatozoa of a boar proven in the IVF system using standard protocols (Hulinska et al. 2011, Anim Reprod Sci, 124: 112–117). The presumptive zygotes were incubated in PZM-3 medium (Yoshioka et al. 2002, Biol Reprod, 66: 112–211) for 15 h, fixed in 2.5% aqueous glutaraldehyde solution (v/v), stained with bisbenzimide-33258 Hoechst (Sigma-Aldrich Co., Prague, Czech Republic) and examined by epifluorescence at a magnification of 400 ×. The proportion of penetrated oocytes from the inseminated ones and proportions of monospermic and polyspermic oocytes from the penetrated ones were assessed. Total efficiency of fertilization (%) of oocytes was calculated according to the formula (ratio of monospermic oocytes (n) to inseminated oocytes (n) × 100). The results were statistically analysed by the ANOVA procedure using the Chi-square test. In MMC-oocytes total efficiency of fertilization increased (51.1, 54.3 and 57.6%) when the oocytes were matured with 0, 4 and 10 mM L-carnitine. Similarly in MLC-oocytes, total efficiency of fertilization increased (42.1 vs 48.8%) in oocytes matured with 4 mM L-carnitine compared to those matured without L-carnitine. On the other hand, total efficiency of fertilization decreased when MLC-oocytes were matured with 10 mM L-carnitine (37.9%). It can be concluded that supplementation of medium with L-carnitine during maturation positively influenced fertilization efficiency of porcine oocytes independently of their meiotic competence. However meiotically more competent oocytes were more capable of utilizing L-carnitine in comparison with meiotically less competent porcine oocytes in which the abundance of L-carnitine had a negative effect on fertilization.

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A202E OPU-IVP and ET

Effects of resveratrol supplementation during *in vitro* maturation and *in vitro* fertilization on developmental competence of bovine oocytes

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Keywords: bovine oocytes, *in vitro* fertilization, *in vitro* maturation, resveratrol.

Resveratrol (3,4',5-trihydroxystilbene) is a phytoalexin - isolated from various plant species, particularly grapevine peel. Recently, resveratrol gained scientific interest because of its strong antioxidant effects it may have health benefits, including protection against cardiovascular diseases. In addition, it has been shown to increase lifespan in several species and activates the SIRT1 gene. The aim of this study was to investigate its effects in bovine early embryo development. We employed three different resveratrol concentrations during *in vitro* maturation (IVM) and *in vitro* fertilization (IVF). Bovine oocytes (n=1648) were collected from slaughterhouse ovaries and subjected to IVM medium supplemented with 0.2µM, 1µM, and 20µM Resveratrol® (Sigma-Aldrich, Buchs, Switzerland) for 24 h followed by IVF with the same concentrations of resveratrol for 19 h. IVM and IVF medium without resveratrol (control) and DMSO supplementation as vehicle control were included in this experiment. Presumptive zygotes were cultured *in vitro* until day 8 to assess embryo development. Maturation rates, cleavage and blastocyst formation were determined. Maturation rates did not differ significantly (0.2µM: 64.2 ± 7%; 1µM: 82.3 ± 4%; 20µM: 68.8 ± 2%; control: 74.6 ± 5% and vehicle control: 70.2 ± 6%, respectively, $p \leq 0.05$) did not differ dramatically. Oocytes cultured in 1µM resveratrol supplemented maturation medium showed distinct detachment of cumulus cells. Cleavage was reduced in the 0.2µM and 20µM group (0.2µM: 44.21 ± 2%; 1µM: 58.4 ± 3%; 20µM: 40.9 ± 5%; control: 56.6 ± 2% and vehicle control: 55.2 ± 6%, respectively, $p \leq 0.05$). Blastocyst development was impaired in the low and high resveratrol concentration group compared to the other groups (0.2µM:11.3±1%;1µM: 28.4 ± 6%; 20µM: 8.2±4%; control: 22.7 ± 4% and vehicle control: 20.8 ± 2%, respectively, $p \leq 0.05$). These preliminary results indicate that very low and high concentrations of resveratrol impair the development to the blastocyst stage. In conclusion, a 1µM resveratrol supplementation during IVM and IVF seems to improve the developmental competence of oocytes, which is reflected not only in elevated blastocyst rates but also in the higher degree of expansion of cumulus cells after IVM and the maturation rates.

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A203E OPU-IVP and ET

Interpretation of equine *in vitro* produced embryo morphology

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Keywords: embryo, Hoechst, horse, ICSI.

To better understand the correlation between equine *in vitro* produced embryo morphology and nuclear status. Oocytes were recovered from abattoir-derived ovaries, matured *in vitro,* subjected to conventional ICSI and cultured *in vitro*. Assessment of nuclear status by staining with Hoechst 33258 and using fluorescent microscopy was performed at the following times after ICSI: Group A) 20 hours to evaluate pronuclear (PN) status; Group B) Day 2, 3, 4, or 7 to determine nucleus number and correlation of morphological cleavage; and Group C) Day 7 to 11 to determine blastocyst development. Only normal nuclei were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Confirmed blastocysts contained > 64 normal nuclei and showed arrangement of an outer rim of nuclei in a presumptive trophoblast layer. Two Day-9 presumptive blastocysts were transferred to the uterus of a recipient mare to evaluate viability. A total of 109 oocytes were subjected to ICSI in groups A and B. In Group A, the rate of PN formation was 43%. In Group B, 64% demonstrated apparent morphological cleavage but only 17% had ≥ 2 normal nuclei on staining and only 6.5% had a number of nuclei that matched the number of visible blastomeres and were appropriate for age. The other stained embryos that appeared cleaved morphologically possessed only degenerated nuclei or were completely anuclear. In Group C (n = 138 injected oocytes), 17 embryos were presumed to have developed to the blastocyst stage based on morphological criteria. Of these, 7 were confirmed blastocysts by staining and 8 were degenerating embryos. One embryo, presumed to be degenerated, was also revealed to be a blastocyst. Notably, as uncleaved oocytes were placed in a separate droplet at Day 4 but were kept in culture, we could evaluate changes in these oocytes over time. Several known uncleaved oocytes increased in diameter on Day 9, which on simple morphological evaluation, could have led to mistaken classification as blastocysts. Transcervical transfer of two Day-9 presumptive blastocysts to the uterus of a recipient resulted in 2 embryonic vesicles detected on Day 14 after ICSI. The smaller vesicle was manually reduced and the remaining vesicle developed normally and is > 250 days gestation. Overall in Group C, including the two transferred embryos, the rate of confirmed blastocyst development per injected oocyte was 7.2%. To the best of our knowledge, this is the first report documenting the morphology and DNA staining of equine *in vitro*-produced blastocysts vs. blastocyst-like structures. Our findings reinforced the importance of removing uncleaved oocytes to limit uncertainty in later assessments of blastocyst development, and of staining embryos for DNA to definitively establish blastocyst development.

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A204E OPU-IVP and ET

Transfer of cattle embryos produced with sex-sorted semen results in impaired pregnancy rate

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Keywords: cattle, embryo transfer, pregnancy, sex-sorted semen.

This study compared the pregnancy rates after transfer of day-7 *in vivo* embryos produced either with conventional or sex-sorted semen from numerous bulls commercially available and extensively used. In addition, mortality of calves born from sexed embryos and conventionally produced embryos was studied. The data consisted of 12,438 embryo transfers, of which 10,697 embryos were produced using conventional semen (CONV embryos) and 1,741 using sex-sorted semen from 97 bulls (SEX embryos), predominantly of Ayrshire and Holstein breeds. Quality codes of embryos were similar in both groups. Of the CONV embryos, 27.4% were transferred fresh, the proportion being 55.7% for SEX embryos. Recipient properties (breed, parity, number of previous breeding attempts and interval from calving to transfer) were similar for both embryo types, heifers representing 57.8% of recipients in the CONV group and 54.8% in the SEX group. Recipients that were not inseminated or did not have a new embryo transferred after the initial one, and had a registered calving in fewer than 290 days after the transfer, were considered pregnant. Data were analyzed with IBM SPSS Statistics, Version 21.The effects of sexing protocol, embryo type (fresh vs. frozen), developmental stage, quality and breed of embryo as well as parity (heifer vs. cow) and breed of a recipient on conception were analyzed using binary logistic regression. Pregnancy rate for recipients receiving CONV embryos was 44.1% and for those receiving SEX embryos 38.8%. The odds ratio for pregnancy in recipients receiving CONV embryos was 1.34 compared with SEX embryos ($P<0.001$). Other factors affecting the pregnancy rate were embryo quality ($P<0.001$), being highest for grade 1 (CONV 45.2%, SEX 42.8%) and lowest for grade 3 (CONV 29.2%, SEX 22.2%) embryos, and developmental stage of an embryo ($P=0.038$). Transfer of earlier developmental stages, i.e. compact morulas, resulted in lower pregnancy rates than transfer of later stages. Also recipient parity affected pregnancy rate ($P<0.001$), the odds ratio for pregnancy for heifers was 1.18 compared with that for cows. There was no effect of the breed on pregnancy rate, neither of an embryo nor of the recipient. The proportion of female calves was 49.6% and 92.3% in CONV and SEX groups, respectively. Calf mortality was 9.0% and 8.9% in CONV and SEX groups, respectively. Mortality of female calves was similar in CONV and SEX groups, 6.6% and 7.7%, respectively. For male calves, mortality was 9.2% in the CONV group but significantly higher, 16.0% ($P<0.05$), in the SEX group. This study showed that transfer of embryos produced with sex-sorted semen decreased the pregnancy rate by about 12% compared with embryos produced using conventional semen. Mortality of male calves born from SEX embryos was higher than for those born from CONV embryos.

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A205E OPU-IVP and ET

Supplementation of culture medium with foetal calf serum or insulin – transferrin – selenium affects the integrity of equine oviduct explants

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Keywords: foetal calf serum, horse, insulin, IVP, oviduct explants, selenium, transferring.

Equine oviduct explants provide an excellent tool to unravel embryo-maternal interactions. They can be cultured *in vitro* for several days in DMEM F12 and serum whilst remaining functionally intact and highly differentiated. However, dark cell degeneration (DCD) has been observed inside the explants (Nelis et al. 2014 RFD 26 954-966). Since serum has been reported to negatively affect cell and embryo culture (Fernandez-Gonzalez 2004 PNAS 101 5880-5885), we aimed to assess the effect of serum and the serum replacer insulin-transferrin-selenium on the prevalence of DCD, ciliary activity, membrane integrity and ultrastructure of equine oviduct explants. Oviducts ipsilateral to the ovulation side were gathered from mares in the early postovulatory stage. Oviduct explants were harvested by scraping and cultured for 6d in 50 µl drops under oil in 5% CO_2 in air in DMEM/F12 (control; Invitrogen, Merelbeke, Belgium), in DMEM/F12 with 10% foetal calf serum (FCS; Greiner Bio-one, Wemmel, Belgium) or in DMEM/F12 supplemented with 5 µg/ml insulin, 5 µg/ml transferring and 5 ng/ml selenium selenite (ITS; Sigma, Schnelldorf, Germany). Three replicates of 60 droplets per condition were performed. With an inverted microscope, every 24h, the percentage of explants with dark zones and the percentage of explants showing ciliary activity were determined. In addition, the percentage of membrane-damaged cells was determined using Trypan blue (Sigma-Aldrich, Diegem, Belgium). At d0, 3 and 6, ultrastructure was assessed by TEM. To compare DCD prevalence, ciliary activity and membrane integrity, binary logistic regression was implemented (SPSS 21 for windows; SPSS IBM, Brussels, Belgium). During the first two days, the prevalence of DCD was significantly lower in the FCS group (36%), when compared to ITS (68%, $P<0.0005$) and the control (67%, $P<0.0005$), indicating an initial protective effect of FCS. From d3 on, significantly more DCD was observed in the presence of ITS and FCS (87% resp. 92%, $P<0.0005$) compared to the control (81%). FCS and to a lesser extent ITS seem to sustain the percentage of explants showing ciliary activity (97%, $P<0.0005$ and 94%, $P<0.0005$) compared with the control (87%). In all groups, as shown by Trypan blue, the explants consisted of >98% membrane intact cells ($P=0.9$). No qualitative differences in the development of DCD was detected by TEM. The outer surface of explants in all groups was highly differentiated and intact. In conclusion, without affecting morphology, components of FCS, which may be depleted after 2 days of culture, turn out to partly protect while ITS enhances the development of DCD. Furthermore, FCS and ITS seem to preserve ciliary activity. Since the toxic margin of insulin and transferrin, but not of selenium, is far above the applied levels in our culture system, amongst others, selenium may play a role in the development of DCD. Further research is needed to unravel the exact cause in the development in DCD in oviduct explants.

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A206E OPU-IVP and ET

Reproductive response of prolific breed and its crosses in intrauterine insemination program

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Keywords: conception, laparoscopic AI, Romanov sheep, synchronization, Turkish native sheep.

The success of laparoscopic AI (LAI) depends on events and factors that interrelate in a complex way. Once the selection and preparation of the ewe have been accomplished, one of the most important steps in the program is the successful synchronization of the ewe to deliver good quality ova to the site of fertilization at a specific time. However, a considerable variation in success rate exists when using this technology whereby conception rates range from 10 to 85%. A major and highly consistent finding from the studies reported showed to be a major difference among the different ewe breeds with respect to pregnancy rate after LAI regardless of the source of that semen. These results confirm the importance of the breed and therefore possible reasons for this effect needs to be elucidated. In this study, we aimed to investigate the reproductive performance of yearling prolific Romanov breed and its half and quarter crosses with Turkish native breeds in a LAI program conducted during the breeding season. In addition, to breed effect we also examined vaginal electrical resistance (VER) values which was reported by the previous researches (Bartlewski et al., 1999; Rezac, 2008) that ewes with lower VER, which means higher estrogen levels. A total number of 30 ewes, equally distributed for each genotype (Romanov: 10, F1 Romanov crosses (F1): 10 and quarter Romanov (Q breed): 10) were included in the experiments. All animals were treated with a vaginal sponge containing 30 mg fluorgestone acetate (FGA; Chrono-gest, Intervet, MSD, Turkey, for 12 d. Immediately following sponge removal, ewes received an injection of 500 IU, i.m. eCG. An experienced laparoscopic AI operator performed the inseminations using fresh diluted semen (100 x 10^6 motile spermatozoa/0,4ml) at 52-55h after sponge removal. The animals were screened for estrus beginning at 24 h after sponge removal and continuing up to 57 h. Animals that did not show any mating marks by 57 h were not inseminated. Electric resistances of vaginal secretions (VER) were measured with a vaginal probe (DRAMINSKI, Poland) that was gently inserted into the vagina prior to LAI. *Conception rate* was determined by *ultrasound* 40 days after AI. The Romanov breed showed the highest estrus response (83%; $P<0.05$)) and, the F1 (40%) and Quarter Romanov crosses (50%) were found similar estrus rates. Conception rates (CR) were 80%, 75% and 57% for Romanov, F1 and Quarter Romanov crosses, respectively ($P>0.05$). Correlation coefficient between vaginal mucous impedance and conception rates was computed as 0.025 and showed to be not significantly correlated with CR. However, compared to F1 and Q ewes Romanov ewes showed more tight VER values which is possibly related to the variation in the moment of estrus.

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A207E OPU-IVP and ET

The application of bovine *in vitro* embryo production technology to develop an in vitro test battery for the screening of estrogenic compounds

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Keywords: 17β-estradiol, bovine IVP, diethylstilbestrol, *in vitro* assays.

The objective of this study was to develop a battery of tests able to identify the two main mechanisms of action of estrogenic compounds: the receptor-mediated mechanism, naturally occurring in hormone-responsive tissues carrying specific receptors (Erα and Erβ) and the direct mechanism through which estrogen and estrogenic compounds bind spindle components and cause a depolymerizing effect on microtubules therefore inhibiting the correct formation of the meiotic spindle. For this purpose two well-known compounds, diethylstilbestrol (DES) and 17β-estradiol (EST), were tested on four different *in vitro* assays: bovine oocyte *in vitro* maturation (bIVM) assay, bovine embryo *in vitro* culture (bIVC) assay and MCF-7 (human breast adenocarcinoma) and BALB/3T3 cell lines (mouse fibroblasts) proliferation and cytotoxicity assays, respectively. For the bIVM assay immature oocytes were aspirated from abattoir ovaries, washed and transferred to oocyte maturation medium, which was supplemented with the test compounds. At the end of maturation the oocytes were denuded, fixed with acetic acid/ethanol (1:3) for 18-24h and stained with lacmoid solution. The completion of meiosis up to the metaphase II stage was considered as the toxicological endpoint. For the bIVC assay, bovine embryos were obtained by IVM and IVF, followed by *in vitro* culture. At day 7 after IVF, embryos were selected at the early blastocyst stage and exposed to test substances from this stage onwards. The toxicological endpoint was the development of embryos up to the expanded hatched blastocyst stage at day 11. For the other two assays MCF-7 cells were cultured in MEM without glutamine and phenol red supplemented with 10% Foetal Bovine Serum (FBS) charcoal stripped, 4 mM α-glutamine and 1 mM pyruvate and BALB/3T3 cells were cultured in DMEM:TCM199 (1:1) supplemented with 10% FBS charcoal stripped. Both cell lines were exposed to test compounds at increasing concentrations. The AlamarBlue® test was performed and data were analysed with a TECAN plate reader (Infinite F200 Pro). Results indicate that only the MCF-7 proliferation assay can detect the receptor-mediated mechanism in the picomolar range of test compounds whereas a cytotoxic effect appeared in both cell lines in the micromolar range of test compounds. Moreover, the bIVM assay can detect the direct mechanism inducing spindle depolymerisation and abnormal nuclear configuration in the range of 1-20 microM. Finally, the bIVC assay does not seem to be informative because only a cytotoxic effect is evident at the highest concentration tested, as for the BALB/3T3 assay. In conclusion, this battery of four tests can allow to discriminate between the two major mechanisms of action of estrogenic and estrogen-like compounds, the receptor-mediated pathway and the direct one.

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A208E OPU-IVP and ET

Using progesterone assay before superovulatory treatment in bovine farms

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Keywords: bovine embryos, progesterone, superovulation.

A French ET team had systematically realized progesterone assay for 15 years to help in the decision to start or not the superovulatory treatment for an embryo flushing in farm.Since 2010, 2210 progesterone assays have been done from 1561 females (1 425 heifers; 1119 Holstein, 30 other dairy breeds, 266 dual-purpose breeds and 146 beef breeds) in 665 farms. The blood samples for progesterone assay were performed by the farmer from 4 to 13 days after a reference heat and sent to a hormonology lab (LNCR, Maisons-Alfort). The superovulation protocol (8 FSH injections) was: D-16 to D-8 = reference heat; D-10 to D-3 = blood sample for progesterone assay; D-2 = input of an implant of norgestomet (Crestar®); D0, 8:00 = first FSH (Stimufol®) injection; D4 = 2 AI depending on heat observation; D11 = embryo flushing. The ET team received the quantitative result from the lab 2 to 6 days after the blood sample. A qualitative result was determined: negative for quantitative result inferior to 1.2 ng/mL, positive for results superior to 1.8 ng/mL and dubious between 1.2 to 1.8 ng/mL. The interval between the reference heat and the first FSH (from 8 to 16 days) didn't influence the number of collected embryos. No clear effect of parity (0, 1, 2, 3, 4 or 5 and more) or kind of breed could be shown, due to the great predominance of Holstein heifers (1 125). Among the 2210 progesterone assays, 1961 (89 %) gave a positive result, 114 (5%) a dubious result and 135 (6%) a negative one. Among the planned embryo collections, 108 (5%) were not performed, 42 due to a negative progesterone result, 66 for other different reasons. The mean progesterone level increased significantly from 4 to 6 days after heat, but this increase was no more significant after 7 days. Actually, 70 % of the negative results were all the same followed by an embryo flushing (result received too late, recipient already prepared…). Of course negative progesterone levels, led to significantly ($p<0.0001$) less total and viable embryos collected than for positive ones: respectively 10.3 ± 8.6 and 5.7 ± 5.3 for positive versus 6.1 ± 5.2 and 3.0 ± 3.6 for negative. But, for the positive results, no effect of the level of progesteronemia on the number of collected embryos has been observed. Because of the very low ratio of embryo flushing finally cancelled due to negative result of the progesteronemia (2%), it has been decided to stop the use of systematic progesterone assay.

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A209E OPU-IVP and ET

Effect of low oxygen tension on mitochondrial activity in cultured pig embryos

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Keywords: IVF, mitochondrial activity, oxygen tension, pig embryo quality.

Mitochondrial membrane potential (ΔΨm) is a key factor of the normal pre-implantation embryonic development due to high correlation with the cellular energy production. Consequently, alteration of ΔΨm may improve performance of pig embryo produced *in vitro*. Therefore the goal of this study was to check whether decreasing of oxygen tension may influence ΔΨm during *in vitro* development of pig embryos. Pig zygotes were collected surgically from superovulated gilts after flushing the oviducts. Zygotes were cultured in NCSU-23 (North Carolina State University-23) medium at 39°C in an atmosphere containing 5% CO_2 and: (A) 21% O_2, (B) 5% O_2, (C) 2% O_2. Embryos at 2- to 4-cell, 8- to 16 cell and morula stages were selected on days 2, 3 and 4 of culture, respectively. To estimate ΔΨm embryos were labeled with 0.5 μM MitoTracker Orange CMTMRos (Molecular Probes Inc.) for 30 min. at 39°C and subsequently analyzed in LSM 510 META confocal microscope (Carl Zeiss GmbH). The amount of fluorescence emitted from the mitochondria in arbitrary unit which proportional to the ΔΨm were measured. Data were analyzed using one-way analysis of variance and post-hoc Tukey test. For zygotes ΔΨm (mean±standard error of the mean) equals 8.07±1.28 (N=19). In group (A) ΔΨm was: 7.74±1.65 (N=17), 14.28±2.45 (N=16) and 15.1±2.44 (N=17) for 2- to 4 cell, 8- to 16 cell and morula stage respectively. In group (B) ΔΨm was: 9.73±0.96 (N=11, 2- to 4 cell), 24.52±2.37 (N=20, 8- to 16 cell) and 28.3±1.33 (N=18 morula). For group (C), ΔΨm was: 10.15±1.19 (N=21, 2- to 4 cell), 26,45±1.88 (N=13, 8- to 16 cell) and 32.57±1,04 (N=21, morula). In all analyzed groups, at the 2- to 4 cell stage ΔΨm was very low with no differences between groups, while significantly increased later, at 8- to 16 cell and morula stage ($p<0.01$). In conclusion, significant differences between embryos at the same developmental stages cultured in different oxygen tension were detected. Mitochondrial membrane potential for 8- to 16 cell and morula cultured at ambient oxygen tension was lower than that of stage matched embryos cultured in hypoxia conditions. Further investigations regarding the oxygen-sensitive hypoxia-inducible factors expression during *in vitro* cultured of pig embryos under different oxygen tensions are required.

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A210E OPU-IVP and ET

Serum-free *in vitro* culture of equine embryos

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Keywords: embryo development, horse, IVP, serum free in vitro culture.

While bovine embryos are routinely cultured in serum-free conditions since serum culture is associated with the occurrence of the large offspring syndrome, equine embryo culture is still conducted in the presence of fetal calf serum (FCS). In the horse, a negative effect of in vitro culture on the foals has not been observed, but early embryonic loss and development of trophoblast-only pregnancies have been associated with in vitro production of equine embryos (Hinrichs et al, Theriogenology 68:521-529, 2007). Therefore, the aim of this study was to evaluate equine blastocyst development and quality in serum-free culture medium. Equine embryos were produced as reported previously (Smits et al. Reproduction 143:173-181, 2012). Briefly, oocytes were aspirated from abattoir ovaries, matured in DMEM/F12 based medium in 5% CO_2 in air and fertilized by piezo-assisted ICSI. Presumptive zygotes were further cultured in DMEM/F12 supplemented with either 1) 10% FCS, 2) 10% serum replacement (SR, Life technologies, Gent, Belgium) and 5 ng/ml selenium, or 3) 0.4% BSA (Sigma-Aldrich, Diegem, Belgium), 5µg/ml insulin, 5µg/ml transferrin and 5 ng/ml selenium (BSA-ITS) at 38.2°C in 5% O_2, 5% CO_2 and 90% N_2. At day 2.5 cleavage was assessed and at day 9 blastocyst rate was evaluated. Subsequently blastocysts were fixed in 2% paraformaldehyde during 20 minutes and stored in PBS with 0.5% BSA at 4°C until staining. Differential apoptotic staining was performed as described previously (Wydooghe et al, Anal Biochem 416:228-230, 2011) to determine total cell number (TCN), inner cell massa/trophectoderm (ICM/TE) ratio and apoptotic cell ratio (ACR). Cleavage and blastocyst rates were compared using binary logistic regression. Data concerning blastocyst quality (i.e. TCN, ACR and ICM/TE ratio) were analyzed using a mixed-model analysis of variance (SPSS statistics 22). Cleavage rates were similar in FCS (22/29, 75.8%), SR (20/28, 71.4%) and BSA-ITS (22/28, 78.6%). No blastocysts developed in the BSA-ITS. Blastocyst rates were not significantly different between FCS (7/29, 24%) and SR (4/28, 14%) and TCN and ICM/TE were not affected either. However, ACR was significantly higher in SR (4.16 % ± 0.49), when compared to FCS (0.88% ± 0.20 , $p<0.001$). In conclusion, serum-free IVC of equine embryos in the presence of SR does not impair embryonic development, but ACR in the resulting blastocysts is significantly increased, when compared with ACR in blastocysts cultured in the presence of FCS.

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A211E OPU-IVP and ET

The effect of dimethylsulphoxide on bovine embryonic development in vitro

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Keywords: cattle, dimethylsulphoxide, embryo development, IVP.

DMSO is routinely used as cryoprotectant or solvent for in vitro production (IVP) of embryos. Based on its high glassforming characteristics it is essential for vitrification but DMSO is also known for its toxicity at higher concentrations. Earlier studies deemed concentrations of up to 0.4% in in vitro maturation and 0.1% in in vitro culture (IVC) as safe with regards to morphological criteria. In the present study, bovine IVP embryos employing standard protocols were exposed to the following DMSO concentrations during IVC: 0% (control group), 0.05%, 0.1%, 0.15%, 0.2% and 0.25%. At day 8 cleavage and developmental rates were recorded. The morphological quality of expanded day 8 blastocyst was assessed with differential cell stainings; live-dead-staining (live-dead ratio) and TUNEL staining (apoptotic index). Fat accumulation was determined by red-oil staining. So far, the following results could be obtained: Cleavage and developmental rates did not differ ($p \geq 0.05$) between embryos of the various groups. Mean cleavage and development rates averaged at 58.3% ± 10.6% and 28.4% ± 9.2% (0 %), 59.5% ± 11.5% and 26.1% ± 7.4% (0,05%), 57.6% ± 6.6% and 21.7% ± 7.1% (0,1%), 58.1% ± 7.8% and 27.8% ± 5.6% (0,15%), 56.6% ± 7.3% and 24.5% ± 7.0% (0,2%), 56.3% ± 10.9% and 23.5% ± 9.9% (0,25%). The live/dead cell ratio was significantly higher ($p \leq 0.05$) in those embryos derived from the 0.1% group [40.1% ± 23.1%] than that from embryos of the other groups [22.6% ± 13.5% (0%), 23.4% ± 10.4% (0.05%), 24.2% ± 14.6% (0.15%), 22.7% ± 14.0% (0.2%), and 20.3% ± 9.9% (0.25%)]. Apoptotic cells in embryos exposed with 0.1 % and 0.2% DMSO were significantly lower than in those of other groups and with 0.05% DMSO the apoptotic cells in this group are also slightly lower compared to those of control group ($p = 0.08$). Apoptotic index was lower in embryos out of the groups supplemented with 0.1% and 0.2 % DMSO compared to those of the control group (0% DMSO: 3.8%±1.6%, 0.05% DMSO: 2.6%±1.6%, 0.1% DMSO: 2.3%±1.8%, 0.15% DMSO: 3.2%±1.5%, 0.2% DMSO: 2.2%±1.5%, 0.25% DMSO: 3.1%±1.7% [$p = 0.09$; $p = 0.06$]). Fat accumulation was significant higher [$p \leq 0.05$] in embryos stemming from the group supplemented with 0.15 % DMSO (0% DMSO: 6616.9 μm^2±2703 μm^2, 0.05% DMSO: 7346.3 μm^2±1981.3 μm^2, 0.1% DMSO: 6975.5 μm^2±1847.9 μm^2, 0.15% DMSO: 9301.1 μm^2±1703.3 μm^2, 0.2% DMSO: 8675.1 μm^2±2271.4 μm^2, 0.25% DMSO: 8300.7 μm^2±2711μm^2). These findings show that DMSO concentrations of 0.1% and 0.2 % used during in vitro culture influences the quality of embryos at the morphological level. However, further analyses to verify these results at the molecular level via RT-qPCR are still needed.

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A212 Embryology, Developmental Biology and Physiology of Reproduction

Parthenogenetic activation, but not electrofusion, alters developmental kinetics and hatching of mouse embryos

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Keywords: blastocyst, electrofusion, parthenogenesis.

The functioning of the trophectoderm (TE) is influenced by epigenetic modifications and requires biparental complementation. Parthenogenesis alters the epigenetic environment and affects the physiological function of embryonic cells. This work aimed to evaluate the development of murine embryos after oocyte and embryonic manipulation. Kinetics and hatching rate were evaluated in blastocysts: *i*) derived from parthenogenetic activation followed (Group EP) or not (Group PG; theoretically haploid) by electrofusion; *ii*) from electrofusion of two blastomers (Group EL; theoretically tetraploid) and; *iii*) from *in vivo* fertilization (Control Group). There was no significant difference (Chi-square or Exact Fisher's Test, $P > 0.05$) for the hatching rate between groups Control and EL (56.9 and 47.5%), but they differed from the other groups. Between the groups PG and EP, hatching rates were similar (respectively, 14.6 and 7.5%) and the lowest of all groups. The electrofusion technique (EL) itself was not deleterious to hatchability. Thus, parthenogenesis itself and/or the activation process might have negatively affected PG and EP groups. There was a clear difference in developmental kinetics between the groups. While group EL developed similarly to the control group, the embryos that underwent parthenogenetic activation were delayed, possibly due to the exclusively maternal genome. There are reports in the literature that it was possible to rescue the paternal imprinting in parthenogenetic mouse embryos, by ESC derivation (Chen *et al.*, Stem Cells, 27:2136-45, 2009), or by serial SCNT (Hikichi *et al.*, Development, 137:2841-47, 2010). The authors reported that parthenogenetic cells could constitute placenta and fetus itself, partially reverting the original imprinting. Although only morphologically evaluated, the difference observed in the embryos of EP group, compared to PG embryos, suggests that diploidy was not beneficial for parthenogenetic embryos as previously described (Liu *et al.*, Biol Reprod, 66:204-10, 2002). We infer that since diploidy on group EP was exclusively maternal, the full function of the trophectoderm was impaired, in which paternal imprinting is important. Expansion and hatching kinetics of blastocysts was used for the assessment of TE functionality. These functions arise from the capacity of TE to pump sodium ions into the blastocoele, promoting water influx. On group EP, the electrofusion apparently was not the detrimental source to the embryos as embryos from group EL had developmental kinetics and hatching rate similar to those in control group.

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A213 Embryology, Developmental Biology and Physiology of Reproduction

Is the count of ovarian antral follicles ≥ 3 mm in diameter associated with fertility in lactating Nelore cows?

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Keywords: antral follicle, fertility, Nelore.

The follicular growth in cattle occurs in a wave pattern of 2 to 3 waves per estrous cycle, and is characterized by synchronous growth of a cohort of antral follicles, from which usually only one of these will become dominant. The amount of recruited follicles per wave is variable among animals and breeds, but is highly repeatable among individuals. This variation in ovarian follicular population can interfere with fertility by influencing oocyte competence (Ireland et al., 2007). We aimed to identify Nelore (*Bos taurus indicus*) cows with high and low antral follicle count (AFC) and to compare their pregnancy rates. We evaluated 268 multiparous Nelore cows from 40 to 70 days postpartum and body condition score between 3.5 to 4.5 (5-point scale). The cows were divided into groups according to the antral follicle count (≥ 3 mm in diameter). Hence, 33% of animals with the greater AFC were enrolled in high population group (HG, n=89, >38 follicles), while the intermediate animals (33%) were allocated in the intermediate group (IG, n=88, between 28 and 38 follicles) and animals (34%) with lower AFC were included in the low population group (LG, n=91, < 28 follicles). The animals underwent three ultrasonographic evaluations (days D-10, D0 and D28). In D0, at random day of the estrous cycle, all cows received an intravaginal device containing progesterone (1.0 g, DIB®) and estradiol benzoate (EB, 2.0 mg, IM, Estrogin®). Eight days later (D8) we administered 75 µg of D-cloprostenol (Croniben®) and the intravaginal device was removed. Twenty-four hours after DIB removal, the cows were treated with EB (1.0 mg, IM) and after 30-36 hours animals were artificially inseminated at fixed-time (FTAI). Data were analyzed using PROC GENMOD and FREQ SAS System 9.1 for Windows (2002-2003). The mean (±SD) of antral follicles in both ovaries was 32.7±17.8. There was no difference (P=0.144) in pregnancy rates between the HG, LG and IG animals (32.6, 46.6 and 42.9%, respectively). But there was a difference in the probability of becoming pregnant (P = 0.0268) decreased as the AFC in anestrus cows increased the probability of pregnancy (N = 138). Thus, we concluded that there was no difference in pregnancy rates between Nelore cows either with high or low population of ovarian antral follicles when submitted to FTAI, however, in this study the animals in anestrus with the lowest AFC were more likely to become pregnant.

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A214 Embryology, Developmental Biology and Physiology of Reproduction

Is the count of ovarian antral follicles ≥ 3 mm in diameter associated with fertility in lactating Aberdeen Angus cows?

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Keywords: Angus, antral follicles, fertility.

Bovine follicular growth occurs in a wave pattern of two or three waves per estrous cycle. These waves are characterized by the growth of a synchronous group of antral follicles from which usually only one becomes dominant. The number of recruited follicles per wave is variable among animals and breeds, but it shows high repeatability within individuals. Ereno et al. (2013) reported that the number of follicles recruited per wave is higher in zebu cattle when compared to taurine. This variation in the ovarian follicular population can interfere with fertility by affecting oocyte competence (Ireland et al., 2007). The objective of this study was to identify Aberdeen Angus cows (Bos t. taurus) of high and low antral follicle count (AFC) recruited per follicular wave and compare their pregnancy rate. We used multiparous cows (272) between 40 and 70 days postpartum and body condition score between 2.5 to 4.0 (5-point scale).The cows were divided into groups according to the antral follicle count (≥ 3 mm diameter). Thus, 35% of the animals with the highest AFC were included in high population group (HG, n=94, >22 follicles), while the intermediate AFC (31%) were placed in the intermediate group (IG, n=85, between 15 - 22 follicles) and animals with the lower AFC (34%) were included in the low population group (LG; n=93, ≤15 follicles). We performed three ultrasound examinations of the ovaries (D-10 days, D0 and D28). At D0, random day of the estrous cycle, all cows received an intravaginal device containing progesterone (1.0 g, DIB®) and estradiol benzoate (EB, 2.0 mg, IM, Estrogin®). Eight days later (D8) we administered 75 µg, d-cloprostenol (Croniben®) and the intravaginal device was removed. After 24 h of DIB removal, cows were treated with EB (1.0 mg, IM) and 30 to 36 h after animals were artificially inseminated at fixed time (FTAI). Data were analyzed using PROC GENMOD and FREQ - SAS System 9.1 for Windows (2002-2003). The mean (±SD) of both antral follicles in all the ovaries was 19.97±9.03. There was no difference (P=0.12) in pregnancy rate among animals HG, LG and IG (54.3, 39.8, and 43.5%, respectively). But there was a difference in the probability of becoming pregnant (P = 0.0491) as increased AFC raised the possibility of becoming pregnant. In this study, we conclude that there was no difference in pregnancy rate after FTAI between lactating Aberdeen Angus cows of high or low population of antral follicles, however, the animals of high AFC were more prospective to get pregnant.

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A215 Embryology, Developmental Biology and Physiology of Reproduction

Accuracy of two forms of early pregnancy diagnosis in cattle by ultrasonography

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UNIFENAS.

Keywords: bovine, pregnancy diagnostic, ultrasonography.

The use of ultrasound in the diagnosis of pregnancy has been an indispensable tool for good reproductive performance. Pregnancy diagnosis via ultrasonography allows early detection of non-pregnant, allow the decision of turning it pregnant quickly, thus reducing the time that these animals do not remain pregnant. There are two ways diagnostic ultrasound for pregnancy: The first is the traditional view of the fetus and heart rate and the second is the observation of the amniotic bladder, characterized by the presence of fully anechoic content and distended uterine wall considered pathognomonic signs of pregnancy in cattle. The objective of this study was to evaluate the accuracy and execution time of these two forms of early ultrasound pregnancy diagnostic in cattle. We evaluated 674 crossbred cows used as embryo recipients in the same rural property, located in southern Minas Gerais. These were distributed randomly in two treatments: T1 (N = 351), diagnosis by detecting the amniotic bladder and T2 (n = 323), by browsing the fetus and heartbeat. All animals were between 28 and 32 days of gestation. The same equipment was used for both forms of diagnosis (Mindray M5 ™) with a transrectal transducer of 5 MHz. The examination of each run time was calculated using a digital timer. The pregnant females in both tests were reassessed by ultrasound considering the characteristics of the bladder and the presence of the fetus 30 days later. The data were evaluated by ANOVA. The differences in the percentage of pregnant females between 30 and 60 were compared using Fisher's exact test. It was considered significant, differences of below that 5% probability. The initial total pregnancy rate was 49.40% (333/674), of which 172 pregnant considered using the T1 and 161, using the T2. The difference between the total number of cows pregnant between 30 and 60 days was 5.70% and 5.88% for T1 and T2, respectively ($P > 0.05$), showing that the two methods have the same accuracy efficiency for pregnancy diagnosis at 30 days. The average running time of diagnosis was lower ($P < 0.05$) in T1 to T2 (0.5 ± 0.3 vs 1.8 ± 1.6 minutes). It is concluded that the two techniques of early diagnosis of pregnancy has the same accuracy after 30 days. As the female time of manipulation is lower in T1, this method should be indicated.

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A216 Embryology, Developmental Biology and Physiology of Reproduction

Reproductive abnormalities in prenatally androgenised male sheep

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Keywords: andronisation, changes, reproduction.

Aims: This study aimed to verify reproductive abnormalities in male sheep exposed in utero to testosterone in a model for polycystic ovary syndrome (PCOS). Materials and Methods: Animals - Adult sheep from Corriedale breed were obtained from a farmer from the state of RS after the approval of Animal Ethics Committee (CEUA – UFSM, number 009-2013). The androgenisation protocol consisted in the administration of testosterone propionate in the mothers (Androgenol®, Juatuba, Brasil) 100 mg i.m. biweekly from the day 30 to 90 of gestation. No treatments were performed in the control group. After the birth of males (controls n=5 and prenatally androgenized PA n=8), they were followed to address the scrotal perimeter and weight. For the semen analysis executed at 16 months, a microscope technique was employed. There was one case of cryptorchid testes in PA group. For comparison between variables with normal distribution, the T Student test was used. Proportions were analyzed by Fisher´s test. A significant p was considered if <0.05. Results: No differences were observed regarding the weight and scrotal perimeter between PA sheep and controls. At 16 months, the weight in control group was (mean + SD) 33.0 ± 2.5 kg while in androgenised male sheep it was 32.2 ± 3.9 kg. Similarly, the scrotal perimeter was at this time 21.3 ± 0.4 cm in controls and 21.1 ± 0.75 cm in PA male sheep (NS).There was one case of cryptorchidism with reduced weight of the testes. The analysis of the semen showed some abnormalities in the androgenized group, where 60% demonstrated vigor equal to one and 75% motility equal or inferior to 40%. However, a significant decrease in the proportion of PA male sheep with these two features together (vigor >2 and motility > 40%) was noted in comparison to controls (p =0.0476, Fisher´s test). Conclusions: Prenatal androgenisation of Corriedale male sheep did not produce differences in the scrotal perimeter although it adversely affected the quality of the semen, as similarly described in Suffolk breed (Recabarren SE et al, Endocrinology 149(12):6444; 2008).

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A217 Embryology, Developmental Biology and Physiology of Reproduction

Evaluation of gestational length in Criollo mares – preliminary data

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Keywords: criollos, equine, reprodutive characteristics.

The participation of Criollo horses in competitions has grown in recent years. The growing appreciation of the Criollo horses market stimulates conducting research about reproductive characteristics of the breed. Despite the growth in the use of reproductive biotechnologies, there are few studies related to pregnancy monitoring in Criollo horses. The aim of this study was to evaluate the physiological variations related to gestational length in Criollo mares. Retrospective and prospective data were collected from a Criollo Farm in the south of Brazil, during breeding seasons of 2008-2014, including 50 mares. The gestational length, age of the mare, number for deliveries, and gender of the foals were considered. The gestational length was determined by the time of the ovulation until delivery. The mares were assigned into two groups according to age: Young mares (until 7 years); and old mares (> 8 years). For the comparison of the gestational length in relation to the age of the mare and gender of the foals was performed Two Sample T test. The Pearson correlation test was performed to evaluate the relationship between the numbers of deliveries and gestational length. Data for response variables were reported as mean + SE. The means of gestational length was 334 days + 1.5 (minimum: 313 days; maximum; 371 days). It was observed shorter gestational length in young mares (331 days + 1.7) compared to old mares (338 days + 2.4). No difference was observed between the gestational length and gender of the foals. It was observed a tendency toward positive correlation between gestational age and the number of deliveries of the mares ($r=0.24$, $p=0.08$). The gestational length in mares ranged from 320 to 360 days, according to the breed and studied population, although pregnancies between 310 until 380 days can be result in healthy foals. These variations occur due to the influence of maternal, fetal and environment factors. Among the maternal factors, we include the age of the mare, nutritional condition and number of deliveries. Fetal factors include gender of the foal and environment factors involve the month of delivery, the weather and the year (Bueno, et al., III Congreso Argentino de Reproduccioón Equina. Córdoba: UniRio, 2013. p. 137-139, 2013). The means of gestational length observed in this study are in accordance with the data described for Criollo breed (Winter et al., J. Equine Vet. Sci. v.27, n.12, p.531–534, 2007). This study is the first description of relationship between the maternal and fetal factors with the gestational length in Criollo mares. We conclude that young mares present shorter gestational length than old mares, and also a positive correlation tendency between the gestational length and number of deliveries in Criollo mares.

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A218 Embryology, Developmental Biology and Physiology of Reproduction

Metabolic evaluation of repeat breeder Holstein cows during summer and winter

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Keywords: dairy cow, metabolism, repeat breeding.

The aim was to determine the occurrence of metabolic changes in blood serum of Holstein repeat breeder cows (RB, n=67) compared to peak lactation cows (PL, n=70) and heifers (H, n=70) in summer (S) and winter (W). The metabolites studied (Automatic Biochemical Analyzer, Randox Daytona) were cholesterol (CHOL), urea, BHB, NEFA, total protein (TP), albumin (ALB), globulin (GLOB), AST, GGT, CK, triglycerides (TRIGS), DHDL, LDL, VLDL, glucose (GLU) and creatinine (CREA). Data was analyzed with GLIMMIX, SAS. There was no season-category interaction for ALB (P=0.58), AST (P=0.10), GGT (P=0.31), CK (P=0.49) and ALB/GLOB (P=0.12), CHOL (P=0.58), LDL (P=0.60), AGNEs (P=0.16), GLU (P=0.88), urea (P=0, 30) and CREA (P=0.34). Also, no category effect for CREA (H: 0.92±0.02; PL: 0.94±0.03; RB: 0.98±0.03mg/dL; P=0.34) or season effect for ALB (3.17±0.03 vs 3.16±0.03mg/dL; P=0.84), CHOL (119.5±6.0 vs 123.6±5.2mg/dL; P=0.31), LDL (78.9±4.9 vs 74.5±4.0mg/dL; P=0.49), GLU (63.5±0.8 vs 62.2±1.1mg/dL; P=0.31) and CREA (0.93±0.02 vs 0.96±0.02mg/dL; P=0.20) in W and S. In S there was greater AST (59.3±3.0 vs 67.5±1.8U/L; P=0.0005) and lower GGT (21.2±3.1 vs 17.8±2.0U/L; P=0.03), CK (140.7±44.8 vs 72.1±4.3U/L; P=0.007), ALB/GLOB (0.77±0.02 vs 0.69±0.01; P=0.002), AGNEs (0.34±0.03 vs 0.29±0.02mMol/L; P=0.03) and urea (33.5±1.3 vs 26.6±0.9mg/dL; P<0.0001), regardless of category. H had higher CK (162.8±61.9b vs 69.7±5.3a vs 80.1±11.1aU/L; P=0.0003) and lower ALB (2.97±0.03b vs 3.24±0.04a vs 3.30±0.03ag/dL; P<0.0001), AST (53.2±1.9b vs 73.4±3.7a vs 64.3±2.6aU/L; P=0.0005), GGT (8.4±0.9b vs 22.1±1.6a vs 28.2±4.9aU/L; P<0.0001), CHOL (60.6±1.5b vs 156.1±5.5a vs 149.5±5.1amg/dL; P<0.0001), LDL (30.2±1.1b vs 102.0±4.4a vs 98.5±4.5amg/dL; P<0.0001) and urea (22.8±1.0b vs 36.0±1.6a vs 30.8±1.1amg/dL; P<0.0001) than PL and RB regardless of season. RB had intermediate values of NEFA (H:0.15±0.01c; PL:0.45±0.03a; RB:0.35±0.02bmMol/L; P<0.0001) and GLU (H:69.9±1.2a; PL:56.4±0.9c; RB:62.0±0.9bmg/dL; P<0.0001). Category-season interaction was found for PT (P=0.03), GLOB (P=0.04), TRIGS (P=0.02), DHDL (P=0.009), VLDL (P=0.02) and BHB (P=0.0003). In W, PT and GLOB did not differ between categories. In S, H were lower than RB and PL for PT (7.5±0.1b vs 7.9±0.1a vs 8.1±0.1ag/dL; P<0.005) and lower than RB for GLOB (4.5±0.1b vs 4.7±0.1ab vs 4.8±0.1a; P=0.0002). For TRIGS and VLDL, H were lower than cows in W (TRIGS: 24.5±1.2a vs 16.3±0.6b vs 16.1±0.9bmg/dL; VLDL: 4.9±0.2a vs 3.3±0.1b vs 3.2±0.2bmg/dL) and higher in S (TRIGS: 27.4±1.4a vs 14.3±0.5c vs 18.0±1.0bmg/dL; VLDL: 5.5±0.3a vs 2.9±0.1c vs 3.6±0.2bmg/dL). Though metabolites values differ between season and category, they are considered within the normal range for dairy cows and are not indicative of pathological changes. Thus, differences must be related to milk production, DMI, physical activity and nutrition inherent in each category/season and cannot be considered as causes of RB.

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A219 Embryology, Developmental Biology and Physiology of Reproduction

Pharmacological blocking of h3k27 trimethylation alters the expression of polycomb repressive complex 2 genes in bovine blastocysts produced *in vitro*

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Keywords: embryo development, h3k27me3, polycomb repressive complex 2.

Trimethylation of histone H3 on lysine 27 (H3K27me3) is established by Polycomb Repressive Complex 2 (PRC2) and it is associated with stable and heritable gene silencing. In pluripotent cells, genes associated with development and cell differentiation are maintained repressed by H3K27me3. However, this process is not fully understood. The AdoHcy hydrolase inhibitor 3-Deazaneplanocin A (DZNep) can block the action of the PRC2enzymes and thereby inhibit H3K27me3. In this study, we evaluated the effect of treating bovine embryos during *in vitro* development with DZNep on the expression of genes encoding PRC2 enzymes (EZH2, EED and SUZ12), and transcription factors regulating cell pluripotency (OCT4 and NANOG) and trophoblast differentiation (CDX2). Oocytes obtained from slaughterhouse ovaries were subjected to *in vitro* maturation (IVM) for 24 h at 38.5°C, with 5% CO_2 in air and saturated humidity. *In vitro* fertilization (IVF) was performed with a previously tested frozen-thawed semen from a single Nellore bull. The oocytes and spermatozoa remained in coculture for 22 h under the same conditions of IVM. In D3 (considering the day of IVF as D0), the cleaved embryos were randomly allocated into four groups and exposed to 5 µM DZNep from: a) D3 to D5 (DZNep D3-D5); b) D3 to D8 (DZNep D3-D8); c) D5 to D8 (DZNep D5-D8); or d) without DZNep (Control Group). Embryos that developed to the blastocyst stage on D8 were collected for RNA extraction followed by qRT-PCR to assess abundance of transcripts. The experiment was repeated three times and all samples were analyzed in duplicate using 30 embryos per group. Total RNA was extracted using the PicoPure RNA isolation Kit (Life Technologies) and cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies). Relative mRNA abundance was normalized to the levels of two reference genes (beta actin and 18S ribosomal). Data were analyzed using ANOVA and the means were compared by Dunnett's test. DZNep treatment did not alter mRNA levels of SUZ12, NANOG, OCT4 and CDX2 in embryos that developed to the blastocyst stage. However, exposure to DZNep from day 3 to 8 increased mRNA levels of genes encoding the Polycomb enzymes EZH2 and EED. Findings from our previous studies confirmed that exposure of bovine embryos to DZNep during these periods of culture reduced blastocyst formation. These findings indicate that inhibition of H3K27me3 alters the regulation of Polycomb enzymes EZH2 and EED in early developing embryos, which suggests that these enzymes are involved in cell proliferation and blastocyst formation in the bovine embryo.

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A220 Embryology, Developmental Biology and Physiology of Reproduction

Meiosis blockage in bovine oocytes with cordicepim: kinetics of maturation and embryo production

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Keywords: meiotic blocker, oocyte maturation, pre-maturation.

The meiosis blockage may be an alternative to improve oocyte maturation *in vitro*. This study aimed to investigate the effects of cordicepim blocker used for the pre maturation of bovine oocytes. To that, three experiments assessed the kinetics of nuclear maturation just after 6 h blocking (experiment 1); after a maturation period of 20 or 24 h (experiment 2), and the in vitro embryo development (experiment 3). Data were analyzed by the chi-square test with 5% of significance level. In experiment 1, 456 oocytes were incubated for 6 h in one of the following treatments: standard maturation medium (containing the additives serum and gonadotropins) without (IVM/CONT) or containing 79.6 nM / mL cordicepim (IVM/CORD), or in TCM-199 medium (without the additives) (TCM/CONT) or in (TCM/CORD) with cordicepim added. Oocytes were fixed for assessment of nuclear status. Cordicepim in the absence of additives (TCM/CORD) blocked significantly more oocytes (67.0%) in VG/VGBD, than the treatments IVM/CONT (52.5%), IVM/CORD (47.7%) and TCM/CONT (45.7%). In the experiment 2, 504 oocytes were submitted to IVM/CONT and TCM/CORD treatments for 6 h, followed by maturation for either 20 or 24 hours. At the end of maturation, oocytes were fixed for nuclear status assessment. The oocytes treated with cordicepim in the absence of additives showed a significant reduction in MII rates (irreversible blockage) after 20 (TCM/CORD+20, 47.3%) or 24 h of maturation (TCM/CORD+24, 64.8%), in comparison to the treatments with additives: IVM/CONT+20 (98.8%) and IVM/CONT+24 (100%). In the experiment 3, 1527 oocytes went through IVM/CONT+20h, IVM/CONT+24, TCM/CONT+20h and TCM/CORD+24 treatments, in order to assess embryo development rates (cleavage and blastocyst) after parthenogenetic activation. Cordicepim significantly reduced the cleavage rates after 20 (42.0 vs. 56.4%) or 24 hours of maturation (44.3 vs. 54.4%). When it comes to the blastocyst rates, cordicepim significantly reduced (12.1 vs. 24.8%) after 20 h of maturation. However, when maturation length was increased to 24 h, blastocyst rates were no longer affected (22.9 vs. 25.0%). We conclude that in the absence of serum and gonadotropins, cordicepim effectively blocks oocytes in an irreversible manner. Conversely, when these additives are present, the blockage does not occur. Furthermore, increasing the maturation length from 20 to 24 h prevents the detrimental effect of cordicepim in the blastocyst rates.

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A221 Embryology, Developmental Biology and Physiology of Reproduction

Characterization of lipidic profile from Piau swine breed

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Keywords: embryo, mass spectrometry, pig.

Piau breed is one of the locally adapted swine breed that is in constant decline of its population, due to absorbent cross with more premature and economically profitable breeds. In this regard, it is important to maintain this genetic source, which can be done by gametes and embryos cryopreservation. However, appropriate techniques for successful cryopreservation of swine germplasm is not established. In order to propose protocols to increase the efficiency of these techniques, it is necessary to know the characteristics of the material to be cryopreserved. Therefore, the aim of this study was to characterize the lipidic profile of Piau embryos. Therefore, Piau gilts had their estrus observed twice a day, and were naturally bred 12 and 24 hours after estrus detection. Six days after the, the embryos was collected by laparotomy. The embryos (expanded blastocyst, grade 1; n = 8) were stored in methanol at -80°C. To determine the profile of phospholipids, spectrums were obtained by MALDI-TOF mass spectrometry. Mass spectrums were acquired in frequency between 700-90 m/z, in positive/reflected mode in an AutoFlex Speed MALDI-TOF/TOF (Bruker Daltonics, Germany) mass spectrometer. For ionization, each embryo was individually allocated in a well of the MALDI plate and covered with a acid 2,5-dihydroxybenzoic acid (DHB) matrix. Sixteen lipids were found. Among the phospholipids, phosphatidyl cholines [PC (32:0) + H]+, [PC (34:1) + H]+ and [PC (36:4) + H]+, represented by 734.5; 760.5 and 782.5 m/z ions, showed high intensity. Some triglycerides were also found: PPL (50:2) + Na+, PPO (50:1) + Na+, PLO (52:3) + Na+ and POO (52:2) + Na+, represented by 753.5; 755.7; 879.7 and 881.6 m/z ions. The lipidic profile found is similar to the embryos and human oocytes spectrums (Ferreira et al Journal of Lipid Research, v.51, p. p.1218-1227, 2010). However, the triglycerides profile observed is different from bovine embryos but similar to bovine oocytes. These results characterize the lipidic profile from Piau and this knowledge can be used to optimize the cryopreservation of Piau embryos, an essential process for the conservation of this genetic material.

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A222 Embryology, Developmental Biology and Physiology of Reproduction

Morphological aspects from placentitis lesions of equine placenta

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Keywords: equine, morphologic aspects, placenta.

Conditions affecting uteroplacental contact and placental efficiency may profoundly influence fetal well-being, development, and survival. In equine, placentitis is the most common condition of placental failure (BUCCA, Vet Clin Equine Pract, 752, 2006). The aim of this study was to evaluate the morphological aspects from placentitis lesions of placenta in Thoroughbreds mares at foaling. The parturition of 188 Thoroughbred mares was assisted during years 2011 to 2013, of which 40 cases of placentitis were identified in histologic evaluation at foaling. The placentas were submited to gross evaluation immediately after expulsion, and fragments were collected from nine placental points (cervical star, uterine body, gravid horn, non-gravid horn, bifurcation, amnion and three points of umbilical cord). The placental fragments were fixated in 10% formalin, to perform histologic slide confection. The slides were evaluated through light microscopic. In 82.5% (33/40) placentas, the gross evaluation were compatible with histologic findings of placentitis, showed areas devoid of villi, edema and suppurated material on the chorionic surface. It was observed that 52.5% (21/40) of placentas showed severe suppurative inflammation throughout the chorioallantoic membrane, with the predominance of neutrophils, necrosis and eosinophilic material consisting of cellular debris present between the chorionic villi. These findings featuring acute placentitis are frequently associated to bacterial infection. In others 47.5% (19/40) placentas the inflammatory infiltrate were formed by mononuclear cells, with a prevalence of macrophages and lymphocytes, mild to moderate necrosis of villi and edema in choriallantois membrane, demonstrate chronic placentitis. Lesions with morphological distribution of ascending placentitis were identified in 72.5% (29/40), these lesions were present in cervical star and uterine body. Focal lesions were observed in 12.5% (5/40) placentas on the regions of gravid horn, non-gravid horn and bifurcation. These morphological characteristics are frequently associated with fungical infection, despite this agent has not been identified on the histologic evaluation. In 15% (6/40), the lesions distribution was diffuse, morphologic characteristic associated with hematogenous infection. The hematogenous placentitis diagnosis can be difficult to perform during gestation, and is frequently observed only in post-partum evaluation, since many mares did not show clinical sings (WILLIAMS, Proc. 22 Workshop on the Equine Placenta, 90, 2004). We concluded that a higher incidence of ascending placentitis in the morphological aspects of lesions was observed. The morphologic characteristics of focal and diffuse lesions were 28%, therefore, more studies are necessary to identified clinical sings and etiologic environment in these cases of placentitis.

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A223 Embryology, Developmental Biology and Physiology of Reproduction

Involuntary culling of dairy cows due to reproductive disorders

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Keywords: dairy cattle, involuntary culling, reproductive problems.

The involuntary culling of dairy cows is a complex practice that depends on the production goals of the farm (Silva et al. Rev Bras Saúde Prod Anim 5(1): 9-17, 2004). This study evaluated the main causes of waste on dairy farms in the central-northwest region of Rio Grande do Sul/Brazil. The data were obtained in a period of five years (2010-2014) from 2861 lactating cows from five farms (A, B, C, D, and E) which a mean of 114.4 ± 17.6 lactating cows/herd. The main causes of involuntary culling related to reproductive, mammary gland or locomotor system were evaluated. The variables analyzed were the MEANS, GLIMMIX PROC, PROC CORR, and PROC REG of SAS (SAS 9.3, USA, 2003). The average milk production/herd during the study period was 23.8 ± 0.6 kg/cow/day (A = 23.5 ± 0.8, B = 23.5 ± 0.7, C = 24.4 ± 0.9, D = 27.6 ± 0.9, E = 20.2 ± 0.7 kg/cow/day) (P < 0.0001). The mean pregnancy rate/AI (P/AI) was 41.5 ± 1.8 (A = 38.3 ± 5.0, B = 42.7 ± 4, C = 42 ± 4.5, D = 35.2 ± 1 6, E = 49.5 ± 3.1% P/AI) (P = 0.1247). Significant negative correlation was found between milk production and pregnancy rate/AI (P < 0.0001; r = -0.79). During the study period 22.2% cows in production activity (i.e. overall discard rate) (634/2861), were discarded. From this group, 77.3% (490/634) were discarded by involuntary culling reason, and 22.7% (144/634) for voluntary discarding. It´s remarkable that the reproductive problems represented the most important cause of culling in herds: 39.4% (250/634), and as well as among the involuntary culling: 51.1% (250/490) (P = 0.001). Diseases of the mammary gland and musculoskeletal system were detected in 38.9% (191/490) and 10% (49/490), respectively. Among the reproductive causes, repeat-breeder cows represented 40.8% (80/196), seropositive for Neospora caninum cows that had abortions: 27.5% (54/196), metritis/ postpartum endometritis: 20.4% (40/196), and 11.2% (22/196) of the cows had others reproductive causes (ovarian cysts, dystocia and obstetric surgery). No differences were observed between farms at the percentage of involuntary culling by reproductive causes (A = 44.2, B = 45.7; C = 48.4 D = 33.1; E = 35.6%) (P = 0.2131). However, the total percentage of discard from farms A and B were smaller (15.7% and 14.6%, respectively); while the farms C, D, and E showed bigger culling rates 24.4%, 25.7% and 24.6%, respectively (P = 0.0129). We concluded that reproductive problems are the leading cause to involuntary culling in the dairy farms of this study. Management improvements and monitoring system for involuntary cutting should be carefully adopted to reduce economic losses.

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A224 Embryology, Developmental Biology and Physiology of Reproduction

Reproductive performance of *Bos indicus* and *Bos indicus* x *Bos taurus* heifers: Effect of eCG and P4 level during TAI programs

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Keywords: *Bos indicus*, *Bos taurus* x *Bos indicus*, TAI.

The aim of this study was to evaluate the effects of P4 level and eCG treatment in the TAI programs for *Bos indicus* (Nellore) and *Bos indicus* x *Bos taurus* (crossbred) heifers. Heifers used in the study (n = 1989) included Nellore (n = 992) and Crossbred (n = 997) that were 14-24 mo of age (BCS: 3.08 ± 0.01, BW: 329.09 ± 0.66 kg). Ovarian ultrasonography was performed twice (7 days apart) on all heifers at the start of the study to identify heifers with a CL present. Heifers with a CL were submitted to a TAI program. Heifers without a CL at either ultrasonography were submitted to a puberty induction protocol (Rodrigues, Theriogenology, 82, 760, 2014). Only heifers with a CL that was detectable by ultrasonography 12 days after puberty induction remained in the study. The TAI program that all heifers received was as follows: D0 – Insertion of an intravaginal P4 device (CIDR 1.9g; 1st and 2nd use = High P4; 3rd and 4th use = Low P4; Zoetis, Sao Paulo, Brazil) and 2 mg (i.m.) of estradiol benzoate (Gonadiol; Zoetis); D7 – 12.5 mg (i.m.) of dinoprost tromethamine (Lutalyse; Zoetis); D9 – CIDR withdrawal and 0.5 mg (i.m.) of ECP (ECP; Zoetis). At this moment heifers were randomly assigned to receive either 0 (Control; 994) or 200 IU (eCG; 995) of eCG (Novormon; Zoetis); D11 – TAI was performed, 48h after CIDR withdrawal. On Days 9 and 11, a subgroup of heifers was evaluated by US in order to record the largest follicular diameter (Ø). Continuous variables were analyzed using the PROC MIXED and the binomial variables using the PROC GLIMMIX, both from SAS. Included in the models were effects of breed, group, eCG and P4 level. Differences were significant when $P < 0.05$. The follicular Ø on D9 was greater for crossbred heifers (10.8 ± 0.01 mm) than Nellore heifers (9.9 ± 0.02) and in heifers from Low P4 (10.7 ± 0.01) when compared to heifers from High P4 (9.9 ± 0.01). The follicular Ø on D11 was greater for heifers from Low P4 (11.8 ± 0.01) when compared to heifers from High P4 (11.4 ± 0.01) and Crossbred heifers tended to have a greater follicular Ø on D11 than Nellore heifers (11.8 ± 0.01 and 11.4 ± 0.01, respectively). Ovulation rate was greater for Nellore compared to Crossbred heifers (91.1% vs 88.0%, respectively). Crossbred heifers (63.0%) had greater conception rate than Nellore (57.8%). Crossbred heifers (58.4%) tended to have greater pregnancy rate than Nellore (54.1%). Furthermore, there was an interaction between P4 level and eCG on pregnancy rate. The High P4 heifers receiving 0 IU eCG (51.9%) had lower pregnancy rate than High P4 heifers receiving 200 IU eCG (62.4%). However, in Low P4 heifers, eCG treatments did not differ (0 IU: 56.6%, 200 IU: 54.1%). Differences between Crossbred and Nellore heifers synchronized with the same TAI program were observed and regardless breed, the eCG treatment increased pregnancy rate in heifers that received high P4.

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A225 Embryology, Developmental Biology and Physiology of Reproduction

Development of bovine embryos *in vitro* in co-culture with mesenchymal stem cells and murine embryonic fibroblasts

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Keywords: in vitro bovine embryo production, mesenchymal stem cells, murine embryonic fibroblasts.

Mouse embryonic fibroblasts (MEFs) have been widely used as a feeder layer to support embryonic stem cells due to their capacity to release growth factors. Mesenchymal stem cells (MSCs) also release bioactive factors which support cell growth. This study aims to investigate the effect of co-culture of MSC from rat bone marrow or MEF as a feeder layer for *in vitro* production of bovine embryos. Oocytes from slaughterhouse were collected and matured (TCM 199 medium in incubator with a temperature of 38°C, 5% CO2 concentration and 95% relative humidity) in control condition (CTRL) or in co-culture with previously inactivated MSC or MEF with 10ug/mL of mitomycin C (Sigma-Aldrich). Fertilization was performed in CTRL condition for all groups, and the embryos were cultured from fourth day in CTRL, or in co-culture with inactivate MSC or MEF, thus the following groups were performed in IVM/IVF: (CTRL/CTRL) - maturation and embryonic culture in CTRL condition; (CTRL/MSC) - maturation in CTRL condition and embryonic culture with MSC; (CTRL/MEF) - maturation in CTRL and embryonic culture with MEF; (MSC/CTRL) - maturation with MSC and embryonic culture in CTRL condition; (MSC/MSC) - maturation and embryonic culture with MSC; (MEF/CTRL) - maturation with MEF and embryonic culture in CTRL condition and (MEF/MEF) - maturation and embryonic culture with MEF. Cell inactivation was performed using mitomycin C. The data was analyzed by chi-square test for oocytes and Kruskal-Wallis nonparametric with Dunn's post-test for embryos. No significant difference in oocytes metaphase II and apoptosis rates and in embryo cleavage rate at 4th day after the beginning of the in vitro culture was found among the oocytes matured in CTRL, MSC or MEF conditions. The rates of blastocyst formation, expanded, hatched and the total of blastocysts did not differ among experimental groups ($P > 0.05$) at 7th day of embryo development. At 8th day of embryo culture we observed a difference ($P < 0.05$) in hatched blastocyst rate which was higher in the CTRL/CTRL group (14.3±1.9%) when compared to MSC/MSC group (3.6±1.4%), however, the proportion of blastocyst, expanded and total blastocysts were not different ($P > 0.05$) among the groups. The number of cells in the inner cell mass, trophoblast cells, apoptotic cells and total cells were similar ($P > 0.05$) in the embryos cultivated at all experimental groups. We conclude that the co-culture in IVM or IVC with MSC or MEF did not affect the bovine embryos development.

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A226 Embryology, Developmental Biology and Physiology of Reproduction

In vitro embryo development and gene expression in granulosa and cumulus cells from *Bos indicus* cows with different numbers of antral follicles

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Keywords: antral follicle population, embryo development, gene expression.

The objective of this study was to investigate if *in vitro* embryo development and gene expression in granulosa cells is affected by the antral follicular population (AFP) in Nelore cows (*Bos indicus*). The average number of AFP was determined in ovaries (n = 336) from 168 Nelore cows, and the ovaries of each cow were identified and kept separately. The mean number of antral follicles was 61.14 ± 30.43 per cow. Ovaries were then separated in three groups as follows: G-High, ≥92 antral follicles; G-Intermediate, 46-76 antral follicles; and G-Low, ≤31 antral follicles. *In vitro* embryo development was assessed using oocytes collected from 752 ovaries of 356 cows in 9 replicates. Oocytes were matured in vitro under standard conditions in groups of 15 oocytes in 100 mL of maturation of medium. Rates of cleavage, development to the blastocyst stage and embryo hatching were compared between groups. Statistical analysis was performed by logistic regression at $P < 0.05$. The cleavage and blastocyst rates did not differ between groups (76.6% [473/617] and 40.6% [251/617] in G-High; 77.5% [457/590] and 36.3% [214/590] in G-Intermediate; 79.5% [418/526] and 38.6% [203/526] in G-Low). The hatching rate was higher in G-High (16.5% [102/617]) compared with G-Intermediate (11.5% [68/590]; $p = 0.0129$) and G-Low (11.6% [61/526]; $p = 0.0179$). Total RNA was purified from granullosa and cumulus cells using Trizol. Quantitation and estimation of RNA purity was performed using a Nano-Drop spectrophotometer, and then 200 ng RNA per sample was reverse transcribed using iScript cDNA synthesis kit (BioRad, ON, Canada). Quantitative Real time PCR was performed on a CFX384TM Real-Time System (BioRad) using iQ SYBR Green Supermix (BioRad). Transcript abundance was normalized to average of the internal control genes RP18S and Cyclophilin. Data were submitted to ANOVA and the averages compared by Tukey's HSD test. There was no significant difference in transcript levels of genes encoding steroidogenic enzymes (CYP19, StAR), cell proliferation and differentiation factors (TGFB1, LIFRa and BMPR2), hormones (AMH), and hormone receptors (FSHr, PGr). These findings suggested that the antral follicular population doesn't affect in vitro embryo development in Nelore cows, as well as the expression profile of genes involved in cell proliferation and follicular growth. However, the higher hatching rate suggests that embryos from high AFP cows have superior quality.

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A227 Embryology, Developmental Biology and Physiology of Reproduction

Different associations between GnRH analogue and prostaglandin for treatments of ovarian cysts in dairy cows

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Keywords: bovine, pathologies, reproductive efficiency.

Ovarian cysts are common in dairy cattle. Successful treatment of the cyst is the regression of the structure and corpus luteum formation (CL) and rapid return to regular estrous cycles. The literature mentions occurrence of cystic structures with luteinized wall, in dairy cows, and the possibility of a beneficial effect on treatment with prostaglandin analogues associated with GnRH. The objective of this study was to evaluate the effectiveness of an analogue of GnRH, the gonadorelin (GO), associated or not with a prostaglandin analogue, sodium cloprostenol (CS), in different protocols for treating cysts. We used 36 Holstein cows with ovarian cysts belonging to four dairy farms located in the southern state of Minas Gerais in which animals were managed in semi confinement. The diagnosis was confirmed by ultrasonography (Mindray® M5), whereas ovarian cysts as anecogenic structure of more than 20 mm in diameter. The animals were randomly divided into five treatments: G1 (n = 16): 2 mL of saline solution (control group), G2 (n = 31), 0.5 mg of GO (Fertagyl® MSD-Brazil); G3 (n = 28): 0.5 mg of GO and 10 days, 0.53mg CS (MSD-Ciosin® Brazil), G4 (n = 29) 0.5mg GO and 0.53mg CS at the same time, and G5 (n = 32): 0.5mg GO and two doses (0.53mg) of CS, the first together with GO and the second 10 days after. The application of cloprostenol along with the GnRH analogue (G4 and G5) aimed to improve the effectiveness of the treatment, mainly for luteinized cysts. The application of cloprostenol 10 days later (G3 and G5) aimed to cause lysis of possible CL formed with pretreatment and acceleration of returning to reproduction. The Treatment was effective when the second evaluation did not detect a cystic structure and luteal tissue mass was found. The Data were assessed for normality. The efficiency of treatments was compared by X2. The average of the different variables were submitted to ANOVA and compared between treatments by Tukey test at 5% significance level. There was no farm effect ($P > 0.05$). The efficiency of treatments was: 18.75%^{a}, 54.84%^{b}, 53.51%^{b}, 79.31%^{c}, 81.25%^{c}, the treatment interval to the first service was: 61.22 ± 17.87^{a}, 44.54 ± 16.44^{b}, 30.87 ± 12.63^{c}, 26.19 ± 14,25^{c}, 18.33 ± 10.18^{d} days and the treatment interval to conception: 71.87 ± 21.85^{a}, 60.76 ± 19.38^{b}, 48.34 ± 16.96^{c}, 46.12 ± 15.61^{c}, 35.07 ± 14.32^{d} days, for groups 1 to 5, respectively. The association between GO to CS at the start of treatment was efficient ($P < 0.05$) to improve the cure rate of the cysts (G4 and G5). The application of prostaglandin analogue 10 days after the start of treatment (G3 and G5) led to faster return of the reproductive activity and conception ($P < 0.05$). It is concluded that the combination of cloprostenol gonadorelin is beneficial in the treatment of ovarian cysts in both situations, i.e. when applied along with the GnRH analogue and also 10 days later.

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A228 Embryology, Developmental Biology and Physiology of Reproduction

Distribution of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area and hypothalamus of cow

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Keywords: gonadotropin-releasing hormone, hypothalamus, neuroendocrinology.

GnRH is the pivotal hormone to control of mammalian reproduction. It is synthesized by hypothalamic neurons and packaged in storage granules that are transported through the axons to the external zone of the median eminence (Seeburg et al., 1987, Rec Prog Horm Res. 43, 69–98). The objective of this study was to characterize the distribution and number of GnRH neurons in the preoptic area and hypothalamus of cattle. Beef cow heads (n=2) were collected from a local slaughterhouse and were perfused through the carotid arteries with 4 liters of heparinized saline solution (5 Units/mL), and 2 liters of 4% paraformaldehyde within 30 min of death. The hypothalamus and preoptic area (25x30x30 mm) of brain were dissected at the Reproduction Research Laboratory of the University of Saskatchewan and fixation was continued 72 hrs by immersion in 4% paraformaldehyde at 4°C. Samples were sequentially dehydrated in 10%, 20%, and 30% of sucrose in Phosphate Buffered Saline (PBS). After each sample sank in the 30% hypertonic solution of sucrose (7 days), tissue block was frozen at - 80°C and sectioned at 50µm thickness using a cryostat microtome from the preoptic area to mammillary area (410 sections). Each section was placed into cryoprotectant solution (30% ethylene glycol and 30% sucrose in PBS with 0.1% of sodium azide), and they were stored in -20°C until further use. Every 20th free-floating section was immunostained for GnRH by incubation in 1:2500 dilution of mouse anti-GnRH monoclonal antibody (EMD Millipore Corporation, Telemuca, USA) for 72 hours at 4°C followed by 1:250 of HRP-tagged goat anti mouse IgG (EMD Millipore Corporation, Telemuca, USA) for 24 hours. Immunoreaction was revealed by using 3,3' diaminobenzidine tetrahydrochloride (DAB, SurModics, Eden Priarie, USA). Specificity of the staining was verified by omitting the primary antibody. Presence of GnRH reactive neuron cell bodies (perikaryon region) and axons was recorded in different regions of the preoptic area and hypothalamus with 10x and 20x objective lens on a Zeiss microscope. A total of 205 perikaryia was identified in the two brain samples. The distribution of GnRH neurons was 43.3% in the preoptic area, 13.4% in the anterior hypothalamus, 41.2% in the medial hypothalamus, and 2.0% in the posterior hypothalamus. There was a high concentration of GnRH positive neurons in the Arcuate Nucleus and Diagonal Band of Broca. Axons were observed in groups or isolated single nerve fiber throughout the preoptic area and hypothalamus, and were detected in a high density in the median eminence. In conclusion, GnRH neurons in cow were accumulated in the Diagonal Band of Broca in the preoptic area, and the Arcuate Nucleus of medial hypothalamus. This study will allow future research to determine the pathways of neuroendocrine control of GnRH secretion.

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A229 Embryology, Developmental Biology and Physiology of Reproduction

Effect of administration of different doses of estradiol followed by progesterone on gene expression of endometrial estrogen and progesterone receptors in non-cyclic recipient mares

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Keywords: endometrial receptors, gene expression, non-cyclic mares.

The aim of this study was to evaluate the effect of administration of different doses of estradiol benzoate (EB) followed by long-acting progesterone (LA P4) on gene expression of endometrial estrogen and progesterone receptors in non-cyclic embryo recipient mares. Fourteen mares were evaluated during anestrus and distributed into two groups: 10 mg BE+P4 (n=7), which received three decreasing doses (5, 3 and 2 mg on consecutive days) of intramuscular EB (Estrogin® - Farmavet), followed by administration of 1.500 mg of intramuscular LA P4 (Sincrogest® - Ourofino) 24 hours after the last dose of EB; and 5 mg EB+P4 (n=7), which received two decreasing doses (3 and 2 mg on consecutive days) of EB followed by administration of 1.500 mg of LA P4, 24 hours after the last injection of EB. As the control group, seven of these mares were reevaluated and used during the cyclic phase. To measure the gene expression of estrogen and progesterone uterine receptors, biopsies were performed immediately before the initiation of treatment with EB (M1), 24 hours after the last administration of EB (M2) and five days after injection of LA P4 (M3). In the control group, biopsies were performed in estrus, when uterine edema (score 2-3) and the presence of ≥35 mm in diameter follicles were detected (M2); and in diestrus, on day five after ovulation (M3). Gene expression analysis of estrogen receptor alpha (ERα), beta (ERβ) and progesterone (PR) were performed by real time RT-qPCR, using beta-2-microglobulin (B2M) as the reference gene. The Wilcoxon signed-rank test for paired data was used to compare relative gene expression between the studied moments. Expression of ERα tended to be higher in 1.88-fold (P=0.06) after administration of EB and in 1.34-fold (P=0.06) after administration of LA P4 in group 10 mg EB+P4, which was not observed in group 5 mg EB+P4 (P>0.05). When gene expression dynamics were compared between groups, there was a 1.21-fold increase in PR expression when M3 was compared to M2 in 10 mg EB+P4 group, which tended to be different (P=0.06) from the 1.73-fold reduction found in the control group when PR expression between diestrus and estrus were compared. No differences were observed when the dynamics of ERα and ERβ expression in M3 in relation to M2 were compared between groups (P>0.05), which were reduced after administration of LA P4 or ovulation. No differences were detected when the mRNA expression of ERα, ERβ and PR from 5 mg EB+P4 group were compared to the control (P>0.05) or 10 mg EB+P4 groups (P>0.05). In conclusion, the administration of 10 mg of EB followed by 1.500 mg of LA P4 was not able to reduce the endometrial gene expression of PR after LA P4 injection, as observed in cyclic mares in diestrus, and the dose of 5 mg of EB followed by LA P4 promoted the most similar ERα, ERβ and PR gene expression changes to those observed in cyclic mares.

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A230 Embryology, Developmental Biology and Physiology of Reproduction

Effect of melatonin on DNA fragmentation and in vitro maturation of bovine oocytes subjected to heat shock

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Keywords: DNA fragmentation, heat shock, *in vitro* maturation.

The aim of this study was to evaluate the effect of different concentrations of melatonin added to the medium IVM in DNA fragmentation and maturation of oocytes subjected to heat shock. Immature oocytes aspirated from ovaries obtained from slaughterhouse were selected and randomly allocated in factorial experiment design 3x2. Three concentrations of melatonin (0 M, 10-6 M and 10-4 M; M5250 - Sigma, St. Louis, MO , USA) added to the medium and two MIV incubation conditions (conventional: 24 hours at 38.5°C and 5% CO_2; or heat shock: 12 hours at 41ºC followed by 12 hours at 38.5ºC and 5% CO_2) were tested, resulting in treatments: M1 (0 M; 38.5ºC; n = 156), M2 (10-6 M; 38.5ºC; n = 154), M3 (10-4 M; 38.5ºC; n = 161), M4 (0 M; 41ºC; n = 154), M5 (10-6 M; 41ºC; n = 143) and M6 (10-4 M; 41ºC; n = 159). The IVM was performed in Nunc plate containing 400 µL of TCM-199 (Tissue Culture Medium 199 - Invitrogen, California, USA) supplemented with 20 µg/mL of FSH (Pluset®, Calier Laboratories, Spain) and 10% of estrus cow serum. After the maturation period, the cumulus-oocytes complex were denuded in a solution of PBS plus 0.1% hyaluronidase (Sigma, St. Louis, USA) by vortexing for 5 minutes and washed twice in PBS containing 0.1% PVP. The oocytes were fixed in 4% paraformaldehyde in PBS for one hour and evaluated by the TUNEL assay (deadend ™ Fluorometric TUNEL System - Promega, Madison, WI, USA) about the percentage of TUNEL positive oocytes (DNA fragmentation) and percentage of nuclear maturation (percentage of oocytes in metaphase II). Four replicates were performed. Data were analyzed by Proc Genmod of SAS software (version 9.1; SAS Institute Inc., Cary, NC, USA) considering effects of repetition, melatonin concentration, incubation conditions and interaction between the factors. Values shown are the mean ± s.e.m. Addition of melatonin did not affect ($P > 0.05$) the percentage of TUNEL positive oocytes (M1 = 2.1% ± 0.7; M2 = 1.9% ± 1.9; M3 = 1.9% ± 1.3; M4 = 5.1% ± 2.7; M5 = 2.3% ± 1.5; and M6 = 1.9% ± 0.7) and there was no interaction between concentration and incubation conditions. Melatonin did not affect the percentage of nuclear maturation in the temperature of 38ºC ($P > 0.05$), however, in the heat shock, the percentage of maturation was higher in M6 treatment when compared to M4 ($P < 0.05$) (M1 = 85.8% ± 2.9^{a}; M2 = 84.0% ± 2.8^{a}; M3 = 79.5% ± 2.9^{ab}; M4 = 61.6% ± 6.9^{c}; M5 = 62.8% ± 8.5^{cd}; M6 = 72.6% ± 5.3^{bd}). The DNA fragmentation was not influenced by melatonin supplementation to the medium MIV. However, there was an increase in the percentage of maturation of oocytes subjected to heat shock in maturation medium with a concentration of 10-4 M in comparison with 0M concentration.

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A231 Embryology, Developmental Biology and Physiology of Reproduction

Cortisol effect and its receptor on bovine embryo production

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Keywords: bovine embryo, glucocorticoid, RNAi.

Cortisol, the main glucocorticoid (GC) found in cattle, acts in several physiological processes, playing a key role at the beginning of pregnancy, because it regulates mechanisms involved in embryo implantation process in the endometrium (Michael; Papageorghiou, Human reproduction update, v 14, p. 497-517, 2008). The main mechanism of GC action is via its interaction with glucocorticoid receptor (GR), which has been identified in bovine embryos. According to this the objective of this study was to evaluate the GC mechanism of action in bovine IVP embryos. In experiment 1 we check if GR is important for early embryonic development. For this we silenced the translation of mRNA for GR, using the RNAi technique. IVP Bovine zygotes were injected with siRNA for GR 16 hours after IVF and were IVC for 8 days until blastocyst stage to analyze embryonic development and quantification of mRNA and protein for GR. Cleavage, blastocyst rates, relative quantitation of mRNA for GR and fluorescence were subjected to ANOVA ($P < 5\%$). The relative quantification of mRNA for GR decreased in 2-4 cell embryos and blastocysts, as in immunofluorescence blastocyst ($P < 0.05$), revealing that there was the translation block. Regarding cleavage and blastocyst rates, they were reduced in the injected group (59.7% ± 5.0, 9.7 ± 1.5%, respectively) compared to control (81.2% ± 9.7; 27 0% ± 8.5, respectively) ($P < 0.05$), furthermore the embryos injected with siRNA for GR were of inferior quality. From the observation that the GR is important for early embryonic development the effect of adding different cortisol concentrations in in vitro embryo culture medium was evaluated in experiment 2. The embryonic development and gene expression (NRF1, COX, TFAM, HSP70, FASN, GLUT1) was assessed. For this CCO were IVM, IVF and subsequently IVC in SOF medium containing 0 (control); 0.01 ug/mL; 0.1 ug/mL or 1 ug/mL cortisol. There was no significant difference in the embryos treated with GC (66.6 ± 6 and 30.5 ± 8.9 to 0.01µg/ mL; 70.0 ± 5 and 35.6 ±10.1 to 0.1 ug/mL; 70.1 ± 11 and 27.7 ± 4.5 to 1 ug/mL, $P > 0.05$) compared to control (67.1 ± 11 and 34.8 ± 9.8) according to cleavage and blastocyst rates, respectively. Because of greater morphological similarities between group of 0.1 µg/mL and the control, this concentration was chosen for analysis of the relative quantification of mRNA. Thus, in vitro produced embryos were incubated from the 1st day with or without 0.1 µg/mL of Cortisol, and on the 8th day were analyzed for gene expression, however there was no difference between groups for any of the transcripts analyzed. Therefore, we conclude that GR is important for the early embryonic development in cattle, however its action is not directly related to interaction with GC.

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A232 Embryology, Developmental Biology and Physiology of Reproduction

Effect of fetal bovine serum in FASN expression in bovine embryos cultivated *in vitro*

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Keywords: embryo, FASN, fetal serum.

Supplementation of the medium with fetal bovine serum (FBS) is widely used to increase the embryo rate, however presents a drawback because it is related to increased intracellular lipid accumulation in embryos (DODE, RBRA, v. 37, 145-150 , 2013). The action of FASN enzyme can be related to this process, since it is responsible for the synthesis of palmitate fatty acid from the precursors malonyl-CoA, acetyl CoA and NADPH (URSTAD-JENSEN, BBA- Mol. Cell Biol. Lipids, v. 1821, 747-753, 2011). Thus, the aim of this study is to evaluate the FASN expression in bovine embryos produced supplemented with different concentrations of FBS. Bovine ovaries were collected at a local slaughterhouse and the cumulus-oocyte complexes (COCs) were matured in vitro in TCM-199 supplemented with 10% FBS, FSH, LH and antibiotic for 22 hours at 38.5°C in 5% CO_2. For IVF, COCs and sptz were co-incubated in TALP medium supplemented with FERT-penicillamine, hipotaurina, epinephrine, heparin and BSA under the same conditions mentioned for IVM. After 24 hours of co-incubation presumptive zygotes were then distributed in droplets cultivation in SOF medium supplemented with BSA and antibiotics according to the experimental groups (0, 2.5, 5 and 10% FBS). For counting the number of total cell, embryos were stained with fluorochrome Hoechst 33342. For the analysis of gene expression, the mRNA was extracted by Trizol® method (CA, USA), subjected to reverse transcription with the help of High- kit Capacity cDNA Reverse Transciption (CA, USA) and then analyzed using an assay relative quantification PCR (kit Power SYBR Green® PCR Master Mix) (CA, USA). Statistical significance was estimated by ANOVA with Tukey's post-test, adopting the significance level of 5%. Regarding the blastocyst rate, a significant difference ($p < 0.05$) was observed between the group without adding FBS (15% ± 7.6) compared to the groups with the addition of SFB - 2.5; 5 and 10% (39% ± 5.9; 37% ± 44% ± 3.9 and 10.2, respectively). With respect to counting the number of embryonic cells, the group supplemented with 2.5% FBS performed better than the non supplemented group with SFB (136 ± 8.5 and 108 ± 12.4, respectively) ($P < 0.05$), however this group did not perform significantly better than the other groups. Furthermore, in the analysis of gene expression, FASN gene regulation was not altered in *in vitro* cultured embryos regardless of the addition of FBS ($P <0.05$). Thus, it is concluded that the culture of bovine embryos with different concentrations of fetal calf serum does not affect FASN gene expression. However, supplementation contributes to embryo development in a quantitative and qualitative way, corroborating previous studies.

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A233 Embryology, Developmental Biology and Physiology of Reproduction

Genetic paternal effects on ovary characteristics and ovarian structures of canchim (*Bos indicus* vs *Bos taurus*) heifers: preliminary data

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Keywords: heifers, physiology, ovary.

The aim of this study was to identify the influence of the paternal genotype on ovarian characteristics of Canchim heifers. Heifers (n=140) were evaluated by transrectal ultrasonography (US; Mindray, DP 2200VET, Shenzhen Mindray Bio-Medical Electronics Co., Shenzhen, China) twice with a 14 d interval to detect the presence of a CL. The presence of a CL was not detected in 45 heifers following evaluations, and 32 heifers were randomly selected (16.0 months; 211.0 ± 3.3 kg) daughters of 6 bulls (A, B, C, D, E and F). Heifers were allocated in a grazing intensive pasture system at an experimental station of the Brazilian Agricultural Research Corporation (EMBRAPA), located in São Carlos, state of São Paulo, Southeast of Brazil. US evaluations were performed every 14 days, from January to April 2015. The ovaries were classified according to their diameter (OC) as: I (< 1.5 cm); II (1.6 cm to 2.5 cm); III (2.6 to 3.5 cm); IV (3.6 to 4.5cm). The largest follicle (LF) and the second largest follicle (SLF) present on the ovaries were also recorded. Statistical analysis were performed using the GLIMMIX procedure of SAS® considering the effects of sire and repetition and the results were presented as least squares mean ± SE. Results were significant when $P < 0.05$. Heifers daughters of bulls A, C and F had greater CO (2.0 ± 0.07, 2.1 ± 0.08 and 2.1 ± 0.05, respectively) than heifers daughters of bulls B, D and E (1.8 ± 0.06, 1.8 ± 0.05 and 1.9 ± 0.06, respectively). Furthermore, heifers daughters of bulls A, C, E and F had greater MF (10.3 ± 0.39; 10.1 ± 0.44; 10.4 ± 0.34 and 10.4 ± 0.29mm, respectively) than the daughters of bulls B and D (0.34mm ± 8.9, 9.1 ± 0.27, respectively). In addition, the SMF of heifers daughters of sires A, C, E and F (5.9 ± 0.31, 6.2 ± 0.36, 6.2 ± 0.28 and 6.1 ± 0.24mm respectively) was also greater than heifers daughters of bulls B and D (5.3 ± 0.28 and 5.4 ± 0.22mm, respectively). This study corroborated data from the literature that showed genotype (sire) effects in reproductive tract characteristics of females and also in the development of ovarian structures.

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A234 Embryology, Developmental Biology and Physiology of Reproduction

Efficacy of half dose of Lutalyse® for reducing progesterone plasma concentrations in bovines

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Keywords: lutalyse, luteolysis, progesterone.

The use of Dinoprost in lactating cows is efficient in inducing luteolysis after 72 hours (P4≤1.0mg/ml) in 91.3% of the animals (Steverson et al., 2010). Based on this data, the aim of this study was to evaluate the efficacy of half a dose of Lutalyse® (Dinoprost Trometamina – Zoetis, São Paulo, Brazil). The study was conducted in 3 commercial farms: the first farm with 54 Holstein heifers (Senador Firmino/MG); the second farm with 108 crossbreed animals (Fama/MG, 54 heifers and 54 cows) and the third farm with 108 Nelore animals (Governador Valadares/MG, 54 heifers and 54 cows). The experiment was carried out the same way for all three farms: after day 6 of the estrous cycle, the animals were randomly divided into 3 groups: 1) Control Group; 2) Lutalyse® full dose group (25mg) and 3) Lutalyse® half dose group (12.5mg). Each group was divided into 2 subgroups (from day 7 to 11 and from day 12 to 17 of the estrous cycle). Behavioral signs of estrus was considered Day 0 of estrous cycle. Two blood samples were collected, the first one immediately before the administration of Lutalyse® and the second one 36 hours after. Based on other studies (Steverson et al., 2010), luteolysis occurred if P4 concentration was higher than 1.0 ng/mL before the administration of Lutalyse and lower than 1.0 ng/mL 36 hours after. Animals with low P4 concentrations (n=9; <0.1 ng/mL) before Lutalyse administration were characterized as non-ovulated and removed from the analysis. Radioimmunoassay was used to measure P4 concentrations and data was analyzed using ANOVA (GLM procedure, SAS Inst. Inc, USA). There was no interaction between treatments and subgroups (P>0.1). No interaction between calving order, breed and treatments on P4 concentration reduction (P>0.1). Average P4 concentration before treatment and 36 hours after was 4.83±0.20 ng/ml and 2.15±0.17 ng/ml (n=261), respectively. P4 concentration before administration did not differ (4.17±0.20 [89]; 4.79±0.24 [87]; 4.99±0.24 [85]; P>0.1) between full dose, half dose and control group, respectively. However, P4 concentration 36 hours after Lutalyse® administration was similar for full dose groups (0.46±0.07 [89]) and half dose groups (0.78±0.12 [85]), and both groups showed lower P4 concentrations than the control group (5.23±0.28 [87]). Overall, a full dose treatment had the same efficacy (90.0% [81/89]) as a half dose (83.7% [81/89]) and both treatments were better in decreasing P4 concentrations than the control group (8.0% [7/87]). Efficacy results were similar among the farms, considering full and half dose groups (Farm 1= 94.4% [17/18] and 88.2% [15/7]; Farm 2= 91.7% [33/36] and 85.7% [30/35] and Farm 3= 88.6% [31/35] and 78.8% [26/33], respectively) and had higher efficacy than the Control group (Farm 1= 11.8% [2/17]; Farm 2= 11.1% [4/36] and Farm 3= 2.9% [1/34]). Half dose of Lutalyse® (12.5mg) had efficiently reduced P4 concentrations 36 hours after the administration.

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A235 Embryology, Developmental Biology and Physiology of Reproduction

The impact of neonatal treatment with a GnRH agonist on reproductive and metabolic endpoints in prenatally androgenised sheep

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Keywords: androgenised, ewes, GnRH agonist.

Aims – The minipuberty (MP) is a transitory period after labor, characterized for the abrupt reduction in gonadotropic axis (for few weeks) which persists until the beginning of the puberty. For its features it may be considered a possible window for therapeutic interventions (Jansen HT et al., Endocrinology 152:4288, 2011). The present study evaluated whether a GnRH agonist (leuprolide acetate i.m.) could change the development of typical features of PCOS such as anovulation and insulin resistance at adult age (19 months). Materials and Methods: Animals – Overall, 49 adult sheep from Corriedale breed were obtained from a farmer from the state of RS after the approval of Animal Ethics Committee (CEUA –UFSM). The androgenisation protocol consisted in the administration of testosterone propionate in the mothers (Androgenol®, Juatuba, Brasil) 100 mg i.m. biweekly from the day 30 to 90 of gestation. No treatments were performed in the control group. Part of androgenized sheep offspring (n=4) received up to 48h after birth 5m i.m. of leuprolide acetate (LA). Along the time, 18 ewes were evaluated: 7 (4 controls and 3 androgenised) were euthanized at 13 months of age, and 11 (5 controls, 3 androgenised and 3 androgenised and treated with LA) euthanized at 19 months of age. Results: Reproductive abnormalities – As expected, the ano-genital distance was higher in androgenized females at birth (mean + SD) of 8.0 ± 0.78 cm in comparison to controls 0.58 ± 0.18 cm ($P < 0.0001$, T Student). Control sheep exhibited a marked ovulatory production of Progesterone along 6 consecutive weeks while androgenized animals shown a clear disruption of ciclicity (66%). No significant changes in the pattern of ovulation could be demonstrated in prenatally androgenized sheep treated with LA. Moreover, evidence of insulin resistance was observed in both androgenized groups through the intravenous glucose test (10 mg/kg) ($P < 0.01$, T Student). Conclusions: According to our preliminar results, the neonatal treatment with LA was not able to avoid the development of metabolic (insulin resistance) and reproductive (at least ovulation) in an animal model of PCOS.

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A236 Embryology, Developmental Biology and Physiology of Reproduction

Roles of cell death in sexual dimorphism during preimplantation development

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Keywords: apoptosis, female embryo, sex dimorphism.

Female bovine embryos progress at lower rates and originate smaller blastocysts than male counterparts. However, when and how sex dimorphism starts to occur is not clear. The knowledge of singularities among female and male embryos can be useful for human assisted reproductive medicine, when X-linked disorders risk is detected, and for livestock sex-specific breeding programs. The aim of this study was to characterize the roles of cell death in development of female and male embryos. Using sex-sorted semen from three different bulls for fertilization, we compared bovine sex-specific embryos at 96, 120 and 144 hpi, assessing quality parameters. For that, embryos with more than 4 cells at 96 and 120 hpi; or more than 8 cells at 144hpi were fixed in 4% PFA and stained for caspase 3 (apoptosis marker) by immunofluorescence. Nuclei were counterstained using HOECHST. Cell fragmentation was estimated by number of enucleated cytoplasm fragments inside zona pellucida. Results were grouped as Female and Male, since consistency among bulls 1, 2 and 3 data was detected. The analysis was performed as follows: I. Total cell number; II. Apoptosis (rate of apoptotic cells in embryos); III. Fragmentation (rate of fragmented cells in embryos). The effect of time over each embryo sex (Kruskall-Wallis/ Dunn, F96xF120xF144; M96xM120xM144) and the effect of sex over each moment (Mann Whitney, F96xM96; F120xM120; F144xM144) were analysed using GraphPad InStat (p=0.05). In this study, 379 embryos (65-93 per group) were evaluated, obtained in three replicates. As expected, mean cell numbers increased from 96 to 144 hpi (F: 11.88±0.53^{a}, 15.42±1.04^{a}, 28.1±2.44^{b}; M: 11.33±0.64^{A}, 16.62±1.12^{B}, 40.19±2.86^{C}). Comparing Female vs Male, decreased cell numbers was detected at 144hpi (F: 28.1±2.44, M: 40.19±2.86*). Regarding apoptosis, in female groups the higher rate was detected at 96hpi (23.08±2.54^{a}, 14.62±2.0^{b}, 14.46±1.94^{b}). For male embryos, at 144 hpi the lowest rate was detected (21.40±2.68^{A}, 15.23±1.63^{A}, 9.71±1.43^{B}). Female embryos presented higher apoptosis rates at 144 hpi (F: 14.46±1.94, M: 9.71±1.43*), in reflex to a cell number decrease and to a tendency (p=0.07) of increase in number of apoptotic cells (F: 2.91±1.50, M: 2.38±1.52). Cell fragmentation remained unaltered for female embryos (17.19±1.67, 15.55±1.55, 14.97±1.34), and for male embryos decreased at 144 hpi (15.76±1.36^{A}, 13.11±1.01^{A}, 10.98±1.19^{B}). Female embryos presented higher fragmentation rates comparing to male group at 144 hpi (F: 14.97±1.34, M: 10.98±1.19*), and this increase was also due to a numeric increase in fragmented cell numbers (3.47±0.22, 2.73±0.17*). These new results lead us to propose that sex dimorphism is established at 144hpi in bovine, during morula-blastocyst transition, and cell death is involved in this process.

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A237 Embryology, Developmental Biology and Physiology of Reproduction

Influence of follicle diameter and time of cleavage on embryo production and profile of histone H3K4 methylation in bovine blastocysts

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Keywords: early cleavage, histone modifications, late cleavage.

The diameter of follicles from which oocytes are retrieved and the time of the first cleavage potentially influence competence and epigenetic reprogramming in early embryonic development. This study aimed to investigate the developmental potential and the occurrence of monomethylation at lysine 4 of histone H3 (H3K4me) in bovine blastocysts of early, intermediate and late cleavage after fertilizing oocytes from small and large follicles. Small (≤ 2 mm) and large follicles (4-8 mm) from slaughterhouse ovaries (n = 1982) were punched. Among the collected oocytes, 699 from ≤ 2 mm diameter follicles and 639 from 4-8 mm diameter follicles were subjected to *in vitro* maturation and fertilization. The presumptive zygotes were cultured, and cleavage rates were evaluated by separating the embryos into early (≤ 28 h post-IVF), intermediate (> 28 h and ≤ 34 h post-IVF) and late (> 34 h and ≤ 54 h post-IVF) groups. The blastocyst rates were further evaluated after 7 and 8 days of culture. The blastocyst and cleavage rates were compared by logistic regression and differences were considered statistically significant at a confidence level of 95% ($P < 0.05$). Among blastocysts, we randomly selected 5 embryos per group and investigated H3K4me by immunofluorescence. The percentage of late-cleaved embryos was higher ($P < 0.05$) than that of early-cleaved embryos for 4-8 mm follicles (late: 30% vs. early: 19%) and ≤ 2 mm follicles (late: 33.8% vs. early: 16.6%), indicating that most embryos start the first cell division cycle later. The blastocyst rate for the 4-8 mm group (36.3%) was higher than that for the ≤ 2 mm group (22.9%, $P < 0.05$). In addition, the blastocyst rates for the early and intermediate cleavage groups (45.3% and 33.8%, respectively) were higher than that for the late cleavage group (13.5%, $P < 0.05$). The blastocysts from all the groups displayed H3K4me staining by immunofluoresence; the staining was particularly intense in the trophectoderm region and was weak or absent in the inner cell mass region. Data from this study demonstrate that higher blastocyst embryo rates are obtained from embryos that cleave within 34 hours after fertilization and from those produced from follicles of 4 to 8 millimeters in diameter, indicating a greater ability of these embryos to develop to the stage of embryonic preimplantation. Furthermore, the presence of monomethylation at H3K4 in all the evaluated blastocysts suggests that this histone modification plays a key role in maintaining embryo viability at this important developmental stage.

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A238 Embryology, Developmental Biology and Physiology of Reproduction

Follicular fluid influence on oocyte competence: identification of factors involved in oocyte quality and embryonic development

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Keywords: follicular fluid, *in vitro* production, oocyte competence.

In the process of *in vitro* production of embryos (IVP), one of the most important stages for embryo development is *in vitro* maturation (IVM), since essential events that influence the quality of the future embryo occur during this phase. The oocyte removal from the follicular environment results in the spontaneous resumption of meiosis, interfering in the oocyte capacitation period *in vitro*. Thus, follicular conditions can result in differences that can later impact the embryo phenotype. The objective of this study was the investigation of fundamental molecules present in follicular fluid (FF) that are possibly involved in oocyte capacitation, embryo viability and competence. For this analysis, bovine ovaries were obtained in commercial slaughterhouse. Follicles of 7-8mm were individually aspirated and Cumulus-oocyte complexes (COCs) with their respective FF (5 follicles/ovary) were separated. *In vitro* maturation of COCs from the same ovary were made in 50µl drops of culture medium using Well of the Well system for 22-23 hours in an incubator at 38.5°C and 5% CO2 and high humidity. Oocyte fertilization was made at the same atmospheric conditions of the IVM for 18 hours, followed by in vitro culture (IVC) until D7 in SOFaa medium containing 5% of FCS, 20 L / mL of essential amino acids and 10 uL / mL of nonessential amino acids. Cleavage and blastocyst rates were evaluated at 40hpi and 168hpi respectively. Glucose, cholesterol and pyruvate molecules present in the FF were quantified by fluorimetric assays using commercial kits and analyzed according to the cleavage and blastocyst rates. The data obtained was then analyzed using the Wilcoxon-Mann-Whitney test (n = 6 replicates) on GraphPad Prism 5.0 software. The results showed a higher glucose level in the FF of cleaved embryos than of non-cleaved ones (CI = 0.234 ± 1.327µM; NCl = 0.554 ± 0.108 µM). Likewise, oocytes that were able to develop into blastocysts were obtained from FF with higher pyruvate and cholesterol concentration (cholesterol - Bl = 33.14µM ±1.98; NBl= 28.86µM ± 1.32), (pyruvate - Bl= 35.83µM ± 2.67; NBl= 28.42µM ± 2.30). These results indicate that glucose can be an important substrate for embryo cleavage and that the presence of cholesterol and pyruvate in the FF is essential for the development to blastocyst stage, thus resulting in higher oocyte quality, which is an essential factor for a better embryo development.

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A239 Embryology, Developmental Biology and Physiology of Reproduction

The effect of insulin-like growth factor-I (IGF-I) on mitochondrial gene expression of bovine oocytes subjected to heat shock

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Keywords: heat shock, IGF-I, mitochondria.

Exposure of bovine oocytes to elevated temperature causes many cellular changes such as increased production of reactive oxygen species and reduced mitochondrial activity. Mitochondrial activity has been shown to be associated with expression of nuclear DNA (nDNA) and mitochondrial (mtDNA) encoded genes. There is evidence that the negative effect of heat shock on the oocyte mitochondrial activity was attenuated by insulin-like growth factor-I (IGF-I) supplementation during in vitro maturation (IVM). Therefore, the objective of this study was to determine the effect of IGF-I on mRNA expression of nDNA (TFAM: mitochondrial transcription factor A and ATP5S: ATP synthase subunit s) and mtDNA (ATP6: ATP synthase subunit 6 and COX1: cytochrome C oxidase subunit 1) encoded genes in bovine oocytes subjected to heat shock during IVM. Cumulus-oocyte complexes (COCs) recovered from slaughterhouse ovaries were distributed in control (22h at 38.5°C) and heat shock (14h at 41°C and 8 hours at 38.5°C) groups in the presence of 0 or 25 ng/mL IGF-I during IVM. After IVM, COCs were mechanically denuded by repeated pipetting for complete removal of cumulus cells. Denuded oocytes were stored at -80°C until RT-PCR. Groups of 30 oocytes per replicate were collected from each experimental group (n = 5 replicates) and submitted to total RNA extraction (RNeasy Mini kit, Qiagen). Reverse transcription (RT) reaction was performed using the Superscript III Kit (Invitrogen). Amplification of target genes was carried out using power SybrGreen® PCR Master Mix. The expression of genes ATP6, COX1, and TFAM ATP5S was determined by real time RT-PCR. Cyclophilin A expression was used as reference gene according to the RefFinder program. Relative gene expression values were obtained by ΔΔCt method corrected by the amplification efficiency for each gene (Pfaffl equation). Data were submitted to least squares analysis of variance using the SAS statistical software. There was no effect of temperature and IGF-I on ATP6, TFAM and ATP5S mRNA expression. Exposure of bovine oocytes to heat shock during IVM increased (Temperature: $P < 0.0005$; Temperature x IGF: $P < 0.01$) COX1 mRNA expression as compared to control. However, supplementation of heat shocked oocytes with 25 ng/mL IGF-I during IVM recovered COX1 gene expression to levels similar to the control group. In conclusion, IGF-I has a regulatory action in COX1 gene expression, possibly acting indirectly on the respiratory chain activity under heat shock.

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A240 Embryology, Developmental Biology and Physiology of Reproduction

Follicular fluid thermoprotective role during *in vitro* maturation of bovine oocytes subjected to heat shock

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Keywords: follicular fluid, heat shock, maturation.

Follicular fluid (FF) is a component of oocyte microenvironment containing plasma factors and specialized molecules secreted by follicular cells and oocyte. Follicular fluid products play an important role in follicular growth and oocyte maturation. Heat stress has been shown to compromise the follicular microenvironment and oocyte maturation. Exposure of bovine oocytes to elevated temperature compromise oocyte maturation and developmental competence. Therefore, the objective of this study was to determine the role of FF on oocyte nuclear maturation and cumulus cells (CCs) expansion in cumulus-oocyte complexes (COCs) subjected to heat shocked. Follicular fluid was collected by aspiration from slaughterhouse ovaries during the winter months, processed and stored at -80ºC. Slaughterhouse COCs were matured in Maturation Medium (MM) (TCM199-Bicarbonate with 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 10 µg/mL FSH, 10 µg/mL LH and 1 µg/mL estradiol 17-b). COCs were distributed in positive control (MM + 10% fetal bovine serum at 38.5ºC for 22 h), 0% FF control (MM + 0% FF at 38.5ºC for 22 h) and heat shock (MM at 41ºC for 14 h followed by 38.5°C for 8 h) in the presence of 0, 10, 15 e 20% FF during in vitro maturation (IVM). After 22h IVM, CCs expansion was evaluated by image analysis (software ImageJ) of each COC before and after IVM (N = 5 replicates using 139-154 COCs/treatment). COCs were vortexed in 100 mg/mL hyaluronidase for 5 minutes to remove cumulus cells. Denuded oocytes were fixed in 3.7% formaldehyde for 30 minutes and stained with 1 mM Hoechst 33342 for 15 minutes to determine meiotic progression (N = 6 replicates using 126–136 COCs/treatment). Data were analyzed by ANOVA (SAS). Heat shock reduced CCs expansion ($P < 0.001$) from 3.38 ± 0.14 (positive control) and 3.00 ± 0.14 (0%FF control) to 1.91 ± 0.14 fold (0% FF heat shock). The proportion of metaphase II (MII) oocytes was reduced ($P < 0.001$) by heat shock from 84.0 ± 4.0% in positive control and 74.9 ± 4.0% in 0% FF control to 46.5 ± 4.0% in 0% FF heat shock. However, addition of 10.15 and 20% FF rescued the deleterious effect of heat shock in CCs expansion (2.62 ± 0.14, 2.63 ± 0.14 and 2.63 ± 0.14 fold for 10.15 and 20% FF, respectively) while the doses of 10 and 15% FF rescued nuclear maturation of heat shocked oocytes (64.5 ± 4.0% and 64.0 ± 4% for 10 and 15% FF, respectively) which was similar to 0% FF control at 38.5°C. In conclusion, addition of 10 and 15% FF to MM rescued oocyte expansion and nuclear maturation of heat shocked bovine oocytes, suggesting that FF factors prevent the deleterious effect of heat shock.

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A241 Embryology, Developmental Biology and Physiology of Reproduction

Profile of mRNA expression of (pro)renin receptor and prorenin during luteinization and luteolysis in cattle

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Keywords: corpus luteum, PGF2α, progesterone.

The effect of renin independent-prorenin on the P4 synthesis in response to LH peak was proposed in the 80s (Sealey et al., Proc. Natl. Acad. Sci. USA, 82, 8705-9, 1986). Recently the (pro)renin receptor [(P)RR], which binds to prorenin and renin, was identified in the bovine corpus luteum (CL). Moreover, our research group demonstrated that intrafollicular blockade of (P)RR decreases P4 plasmatic levels during the initial process of luteinization. The aim of this study was to evaluate the profile of mRNA expression of prorenin and (P)RR during bovine luteinization and luteolysis. Thirty cyclic cows of European breed were synchronized with an injection of 500µg of sodic cloprostenol (PGF2α-IM). The estrus was observed and the ovulation was monitored by ultrasonography. The animals were randomly ovariectomized on days 5 (n=4) and 10 (n=5) after ovulation to characterize prorenin/(P)RR during CL formation. To study the profile of mRNA expression of prorenin/(P)RR during luteolysis, cows with CL of 10 days (0h) of estrus cycle received an injection of 500µg of sodic cloprostenol (PGF-IM) and were ovariectomized at 2 (n=3), 12 (n=3), 24 (n=4) e 48h (n=4) after PGF2α injection. The in vivo model was adapted from Shirasuna et al. (Domest. Anim. Endocrin., 43, 227-238, 2012) and confirmed by serum P4. The CL tissue was submitted to Trizol® (Invitrogen, Carlsbad, CA) protocol to extract total RNA, which were quantified by spectrophotometer (NanoDrop, Thermo Scientific, USA). The total RNA was treated with DNase (Promega, Madison, WI) and transcriptase reverse reaction was performed with iScript (Bio-Rad, Hercules, CA), according to the fabricant instructions. The genic expression was evaluated by qPCR and the variability in the quantification of mRNA was evaluated in relation to GAPDH. The results of mRNA expression of prorenin and (P)RR were evaluated by multi-comparison of means test least squares means (LSMEANS). All continuous variables were tested to normality using Shapiro-Wilk test and when necessary normalized. On the 10 day after ovulation (0h after PGF2α), the expression of prorenin mRNA (5.17±2.73) and (P)RR (1.99± 0.57) was significantly increase compared to 5 day of the estrous cycle (0.57±0.21 e 0.53± 0.12 respectively; $P<0.05$). After PGF2α treatment, results of mRNA expression of prorenin and (P)RR suggested a decrease in all hours tested (3.72±2.83; 3.43±0.84; 2.18±0.52; 1.02±0.12 e 1.56±0.56; 1.48±0.17; 1.06±0.10; 1.49±0.44, respectively) compared to 0h, except 12h after $PGF_{2\alpha}$. In conclusion, our results evidence the presence of prorenin/(P)RR in the bovine CL and suggest a major involvement on luteinization than in the luteolysis.

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A242 Embryology, Developmental Biology and Physiology of Reproduction

Plasma progesterone profile and luteal characteristics in pregnant and non pregnant Saanen goats

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Keywords: cavity corpus luteum, estrous cycle, ovary.

The aim of this study was to associate the plasma progesterone profile (P4) in pregnant and non-pregnant Saanen does with the number of corpora lutea (CL) and presence of luteal cavity in an entire estrous cycle. A total of 23 does (64.1 ± 5.4 kg / 3.3 ± 0.4 BCS / 2.8 ± 1.4 years old) had their estrous synchronized, during the breeding season, using two doses of 37.5 ug d-cloprostenol (Prolise®, Tecnopec LTDA, São Paulo, Brazil) at seven days interval. After estrus detection, all does were mated. From the first day of the estrous cycle (day after ovulation), daily, blood was sampled to P4 measurement and ultrasound monitoring (Sonoscape®, Shenzhen, China) of the luteal dynamics was performed until luteolysis and subsequent ovulation or pregnancy at 21 days. Plasma P4 was assessment with a commercial solid phase radioimmunoassay (RIA) kit (Beckman Coulter®– Immunotech, Marseille, France). Data were analyzed by ANOVA, followed by Bonferroni test ($P< 0.05$). A total of 50 CLs from 10 pregnant does and 13 non-pregnant were assessed. Non-pregnant females showed greater frequency of solitary CLs (83.3%) than pregnant ones (16.7%). No difference between the presence of two (54.5% vs. 45.5%) or three (33.3% vs. 66.7%) CLs, as well as the presence or absence of luteal cavity (54.0% vs. 46.0%) on the pregnancy rate was observed. There was no effect of the number of CLs and the presence of luteal cavity on the plasma P4 in does that became pregnant or not. An effect in the day of estrous cycle and interaction between day x pregnancy on P4 values was found. Pregnant does had different plasma profile from 16th day of the cycle (16.7 ± 4.8 vs 10.2 ± 5.8 ng/mL) compared to non-pregnant does. Within the pregnant does group, a stabilization and maximum P4 values from the 8th day of the cycle (12.8 ± 2.2 ng/mL) to the 21st day was observed, with concentrations averaging 13.6 ± 2.9 ng/mL throughout time. In the non-pregnant group, a stabilization and maximum P4 values from the 6th day of the cycle (9.8 ± 2.8 ng/mL) to the 16th day was found, with concentrations averaging 12.3 ± 3.8 ng/mL throughout time. Furthermore, there was an intense hormone drop from the 17th day (5.9 ± 5.3 ng/mL) to achieve baseline in the 21st day (1.1 ± 0.8 ng/mL). In conclusion, non-pregnant does showed greater frequency of solitary CL. Although no difference in P4 values on the number of CL and presence or absence of luteal cavity in the physiological state of the doe were found, pregnant does demonstrated stabilization and maximum P4 production later in comparison with non-pregnant females.

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A243 Embryology, Developmental Biology and Physiology of Reproduction

Main causes of abortion in beef herds in southern Brazil and Uruguay

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Keywords: *Leptospira sp*, reproductive management, *Ureaplasma sp*.

The pregnancy losses represent a significant in genetic and economy in the industry, that often leads to involuntary culling of females. Especially, if the cause comes from a contagious disease to other animals and/or humans (Grooms, Vet Clin North Am Food Anim Pract 20:5-19, 2004). The aim of this study was to determine the main causes of abortion in beef herds from two farms with an extensive management system. The herd with 10.832 *Bos taurus taurus* and mixed animals (A) was located in Uruguay, Melo/Cerro Largo. The other farm located in Cachoeira do Sul, RS/Brazil had 3.280 animals *Bos taurus taurus* (B) and neither of the farms used vaccines for diseases in their herds. The animals were separated by categories (nulliparous, primiparous, multiparous) and according to their reproductive management: artificial insemination (AI) and natural mating (NM). Blood samples from the farm A (152 females and 16 bulls) and B (90 females and 14 bulls) were collected. The serum was used for diagnosis of *Leptospira* sp with microagglutination test (MAT), and vaginal swab or preputial wash for *Ureaplasma* sp identification with nested-PCR. The statistical analysis were performed using MEANS e PROC GLIMMIX from SAS (SAS 9.3, USA, 2003). On the farm A: 57.2% (87/152) of females and 87.5% of bulls (14/16); and in the farm B: 78.9% (71/90) of females and 57.1% (8/14) of bulls were MAT-positive serum for *Leptospira* sp. *Ureaplasma* sp was identified on 28.3% (43/152) of females and 62.5% (10/16) of the bulls from farm A, and 40.0% (36/90) of the females and 57.1% (8/14) of bulls from farm B. The serotypes identified most frequently were *L. pomona* 87.2% (157/180) and *L. hardjo* 37.7% (68/180). Other serotypes with concomitant and a lower serology percentage were: *L. icterohaemorrhagiae*, *L. butembo*, *L. bratislava* and *L. canicola*. The abortion rate in MAT-positive serum females for *Leptospira* sp was 64% (101/158) and 25% (21/84) for MAT-negative serum females. *Ureaplasma* sp was responsible for 70.9% (56/79) of abortion (P = 0.0169). The abortion rate in seronegative females to Leptospirosis and negative for Ureaplasma was 6.6% (3/45). Higher abortion rate occurred in nulliparous, 67.5% (27/40), while on primiparous was 49.2% (31/63), and 46% (64/139) on multiparous. The management practice type did not interfere in the rate (P = 0.8242), because natural mating was responsible for 54.9% (84/153) of the abortions and artificial insemination for 42.7% (38/89). The advantage of using the AI was shown here by the lowest percentage of positive females for Ureaplasma, 4.5% (4/89), while females submitted to the NM had 49.0% (75/153) (P = 0.0001). Regarding the number of calvings, 84.6% (11/13) of nulliparous aborted. Furthermore, *Ureaplasma* sp and *Leptospira* sp led to significant gestation losses in both herds.

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A244 Embryology, Developmental Biology and Physiology of Reproduction

Prorenin blocks forskolin effect and resumes meiosis in bovine oocytes

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Keywords: cAMP, cGMP, *cumulus* and oocyte cell.

Prorenin levels are increased in follicular fluid after LH surge. Recently, our group identified the presence of (pro)renin receptor [(P)RR] in bovine cumulus-oocyte complex (COC). Our aim was to evaluate the effect of prorenin in resumption of meiosis and in the levels of cAMP and cGMP in cumulus cells (CCs) and oocytes. For this, bovine ovaries were obtained in a local abattoir and approximately 20 COCs/ treatment were cultured in 200µl of TCM-199 for 15 hours at 39°C. The treatments were: positive control, negative control (FSK; 200µM), FSK and prorenin (10-10M), and FSK, prorenin plus aliskiren (ALK; direct renin inhibitor, 10-7M). Nuclear maturation was considered when oocytes reached the stage of metaphase I (MI) using10mg/mL of bisbezimide (Hoechst 33342) in fluorescence microscope. Statistical analysis was performed using SAS with significance of 5%. The rate of oocytes that reached MI was greater in the group treated with FSK plus prorenin (38.39%) compared with negative control (18.92%), and FSK, prorenin and ALK groups (8.68%, $P<0.05$). To determine the effect of prorenin on cAMP and cGMP concentrations in oocytes and CCs, COCs were cultured for 6 and 15 hours under the same conditions previously described and distributed as follows: positive control, negative control (FSK), prorenin and FSK plus prorenin. The concentrations of cAMP and cGMP were measured on CC (n=60 COCs) and oocytes (n=50) after 6h of culture using cAMP EIA kit (No. 581 001; Cayman Chemical) and cGMP EIA kit (No. 581 021; Cayman Chemical) according to manufacturer's instructions. Data were tested for normal distribution using the Shapiro-Wilk test and normalized when necessary. Variables from different treatments were compared by ANOVA. Nuclear maturation from COCs cultured by 15 hours were considered as controls. Oocytes treated with FSK plus prorenin reached higher MI percentage (49.95%) than negative control (25.59%; $P<0.05$), however lower percentage than positive control (83.12% MI) and prorenin (78.34%) groups. Intra-oocyte cAMP concentrations were slightly reduced in COCs treated with prorenin plus FSK (8.66 ± 1.20) compared to negative control (10.33 ± 0.88). The positive control (4.00 ± 1.52) and prorenin (3.00 ± 0.57) groups showed lower concentration of cAMP compared to the negative control. Furthermore, concentration of cAMP (15.08±6.7, 9.91±3.68, 2.02±1.43 and 5.05±4.66) and cGMP (0.42±0.19, 0.31±0.07, 0.78±0.4 e 3.61±2.96) in cumulus cells and cGMP in oocytes (0.57±0.09, 0.71±0.09, 0.78±0.12 e 0.60±0.01) did not differ following the treatments (FSK, FSK plus prorenin, positive control and prorenin, respectively). In conclusion, our results indicate that prorenin acts on resumption of meiosis in bovine and suggest a regulation in the concentration of intra-oocyte cAMP.

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A245 Embryology, Developmental Biology and Physiology of Reproduction

Natriuretic peptide receptor 3 (NPR-3) is negatively regulated by LH + FSH in bovine cumulus cells

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Keywords: cumulus oophorus, expansion, natriuretic peptides.

The natriuretic peptides (NP) system consists of three distinct endogenous peptides: A-type NP (ANP), B-Type (BNP) and C-type (CNP), and three receptors: NP-1 receptor (NPR-1), NPR-2 and NPR-3. It has been demonstrated in mice (Zhang et al., 2010, Science, 330, 366-369) and pigs (Zhang et al., 2015, J Cell Physiol, 230, 71–81) that CNP produced by mural granulosa cells, binds to NPR-2 in cumulus cells and maintains the oocyte arrested at germinal vesicle stage. After the LH surge, expression of CNP in granulosa and NPR-2 in cumulus cells declines (Kawamura et al., Hum. Reprod. 26, 3094–3101). However, the role of NP receptors on meiotic regulation has not been systematically investigated in monovular species. Previous studies from our laboratory, using cattle as an experimental model, revealed an increase in CNP expression in granulosa cells after the LH surge. Moreover, we found that the three NPs stimulate meiosis resumption and cumulus cell expansion in cumulus-oocyte complex (COC) cultured with forskolin. The aim of this study was to evaluate the expression kinetics of NP receptors during in vitro maturation of bovine COCs cultured with or without gonadotropins. COCs were collected from ovaries obtained from a slaughterhouse, selected (quality 1 and 2) and cultured in TCM 199 for 3, 6, 9 and 12h, with or without FSH (0.5mg/mL) and LH (5.0µg/mL). In each time point, an image was captured from 10 COCs of each treatment and the total area was measured (µm2/COC; LeicaApplication) to evaluate cumulus expansion. To evaluate meiotic progression and expression profile of NP receptors in cumulus cells, 30 oocytes from each treatment and time point were used. The experiments were performed in quadruplicate. Transcript levels were quantified by qPCR and differences between groups determined using LSMean and Student's t test with 5% significance. We observed that up to 6 h of culture, over 80% of the oocytes remained in germinal vesicle in both groups. However, at 9 h and 12 h of maturation, meiotic resumption was observed in 86.9% and 99.0% of oocytes in the gonadotropin group, and 56.3% and 70.4% in the control group, respectively. In addition, we observed that COCs of the control group had similar cumulus area at 0 h during 12 h of culture. Contrarily, a three-fold increase in the COCs area was observed from 6 h to 12 h of maturation in the gonadotropin group. There was no difference in the transcript levels of NPR-1 and NPR-2 between treatments or time in culture. However, NPR-3 mRNA levels in cumulus cells decreased from 0 h to 9 h in the gonadotropin group. In the control group the highest levels of NPR-3 mRNA were detected at 12 h of culture. These findings indicate that NPR-3 expression is negatively regulated by FSH+LH during in vitro maturation of bovine COCs.

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A246 Embryology, Developmental Biology and Physiology of Reproduction

Dynamic remodeling of endometrial extracellular matrix regulates embryo receptivity in cattle

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Keywords: cattle, endometrium, receptivity.

We aimed to evaluate in the bovine endometrium whether (1) key genes involved in endometrial extracellular matrix (ECM) remodeling are regulated by the endocrine peri-ovulatory milieu; and (2) specific endometrial ECM-related transcriptome can be linked to pregnancy outcome. In Experiment 1, pre-ovulatory follicle growth of cows was manipulated to obtain two groups with specific endocrine peri-ovulatory profiles: the Large Follicle Large CL group (LF-LCL) served as a paradigm for greater receptivity and fertility and showed greater plasma pre-ovulatory estradiol and post-ovulatory progesterone concentrations when compared to the Small Follicle-Small CL group (SF-SCL cows). Endometrium was collected on days 4 and 7 of the estrous cycle. Histology revealed a greater abundance of total collagen fibers in SF-SCL on days 4 and 7 endometrium. In Experiment 2, cows were artificially inseminated and, six days later, endometrial biopsies were collected. Cows were retrospectively divided into pregnant and non-pregnant (P vs. NP) groups after diagnosis on day 30. In both Experiments, expression of genes related to ECM remodeling in the endometrium was studied by RNAseq and qPCR. Gene ontology analysis showed an inhibition in the expression of ECM-related genes in the high receptivity groups (LF-LCL and P). Specifically, there was downregulation of TGFB2, ADAMTS2, 5 and 14, TIMP3 and COL1A2, COL3A1, COL7A1 and COL3A3 in the LF-LCL and P groups, and this was confirmed by qPCR. Results suggest receptivity is associated with a tight control of the abundance of ECM components and that disregulation could perturb the initial embryonic contact with maternal endometrial tissue leading to failure of pregnancy. In summary, the overlapping set of genes differently expressed in both fertility models: (1) suggests that disregulation of ECM remodeling can impair receptivity and (2) can be used as markers to predict pregnancy outcome in cattle.

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A247 Embryology, Developmental Biology and Physiology of Reproduction

Transfer of two demi embryos increases pregnancy rate but not the birth rate

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Keywords: embryo, gestation, micromanipulation.

This study aimed to compare the viability of bovine demi-embryos inovulated alone or in pars versus intact embryos. Twenty five Simmental and Aberdeen Angus cows were used as embryo donors, and 153 crossbred heifers were used as recipients. Donors were superovulated by conventional protocol with eight decresasing doses of FSH; and the embryos were collected by nonsurgical technique. Only excellent embryos morphologically (G1) classified as morulae, early blastocyst and blastocyst stage, were split. Seventy eight embryos were submitted to bisection by using a micro surgical blade, and 52 were kept intact. Embryos were transferred into the recipients in three treatments: T1 (intact embryos; n = 52 recipients); T2 (1 demi-embryo/receptora; n = 54 recipients); T3 (2 demi-embryos; n = 51 recipients). Recipients were synchronized by a single injection of sodic cloprostenol. Embryos and demi-embryos were non-surgically inovulated 6 to 8 days after estrus in the uterine horn ipsilateral to the corpus luteum. Pregnancy diagnosis were done at 30 and 60 days of gestation. The means of gestation rate were compared by $\chi 2$. An economic analysis was performed considering the costs of the: recipients, the embryo production and transfer (i.e., hormones, disposables materials, handling media, semen, and the hand-to-work). The inovulated cows pregnancy rate did not differ among treatments ($P>0.05$) at 30 (55.8; 47.1 and 62.0%) and 60 days (51.9; 37.3 and 54.0% for T1; T2 and T3, respectively). Pregnancy rate per original embryo was greater in T2 (88.9%) than T1 (55.8%) and T3 cows (60.8%) ($P < 0.05$) at 30 days, however, there were no differences ($P > 0.05$) among treatments at 60 days (51.9; 70.4 and 52.9 for T1; T2 and T3 cows, respectively). The percentage range of twin pregnancies was 0 – 0%; 1 – 5.3% and 10 – 37.0% for T1; T2 and T3, respectively. The means percentage of live born calf by inovulated recipient did not differ (48.1; 31.4 and 34.0% for T1; T2 and T3, respectively). The percentage of live born calf using one original embryo was better ($P > 0.05$) in T2 (59.2%) than T3 cows (33.3%). The abortion rate was higher in T3 cows ($P < 0.05$). The mean cost of calf born alive was U$287.3; 262.5 and 385.4 for T1, T2 and T3 cows, respectively. It is concluded that bisection and transfer of one demi-embryo do not reduce costs of calf born alive. Transfer of two demi-embryos into the same recipient did not improve pregnancy rate.

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A248 Embryology, Developmental Biology and Physiology of Reproduction

Intrauterine treatment in repeat breeder dairy cows: preliminary data

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Keywords: endometritis, PMN, uterus.

Postpartum uterine infections have a high prevalence and a negative effect on reproductive performance (RP) in dairy cattle. The meta-analysis of the efficacy of treatments for endometritis with PGF2α (Heimerl & Heuwieser J Dairy Sci 96, 2973-2987, 2013) revealed large discrepancies between results. In the present study the impact of endometritis on the RP of repeater breeder dairy cows (n = 30) with chronic postpartum endometritis was evaluated after: uterine infusion of saline solution (500ml) + sodium cloprostenol IM (25mg, Sincrocio®, Ouro Fino , Brazil) (n = 10; SFPG), uterine infusion of ceftiofur (500mg, Ceftiomax®, Biogenesis Bago, Brazil) (n = 11; CPG) or sodium cloprostenol IM (25mg, Sincrocio®, Ouro Fino, Brazil) (n = 9; PG). Gynecological examination and ultrasonography were performed in all females. To establish the presence of endometritis, a endometrial cytology (Cytobrush, Minitube, Brazil) was done according to Barlund et al. (Theryogenology 69, 714-723, 2008) (PMN1) in the uterine body, and the cutoff point was the presence of ≥7% of polymorphonuclear neutrophils (PMN) (Gilbert et al. Theriogenology 64, 1879-1888, 2005). The slides were stained with fast panoptic. Cytological evaluation determined the percentage of neutrophils (%PMN), counting at least 200 cells under optical microscopy (400X) for the quantitative evaluation of endometrial inflammation. Repeat breeder cows with more than three AI above 100d of postpartum, and ≥7% of PMN were included in the study. Ten days after treatment new cytology was obtained as the first examination (PMN2). The AI was conducted after estrous detecting and RP observed during the three subsequent cycles. Ultrasonography examination was done 28d after AI in order to detect the early pregnancy (P/AI). Statistical analysis was performed using PROC GLM and PROC GLIMMIX from SAS (SAS 9.3, USA, 2003). The average DIM was 209.6 ± 13.9 days (SFPG = 227.4 ± 31.8; CPG = 197.1 ± 0.4; PG = 219.4 ± 28.4; P = 0.7213), the number of AI was 4.4 ± 0.31 AI/cow (SFPG = 4 ± 0.5; CPG = 4.7 ± 0.6; PG = 4 ± 0.53; P = 0.716) and the average production was 26.5 ± 1.4 kg milk/cow/day (SFPG = 27 ± 3; CPG = 27.4 ± 2.1; PG = 25 ± 1.3; P = 0.5902). In PMN1 the average was 7.07 ± 0.7 and 0.99 ± 0.25 after, and showed significant effect (P = 0.002) in reducing PMN after treatment [(SFPG: 8.6 ± 0.8% vs 0.6 ± 0.4%), (CPG: 8.5 ± 0.6% vs 0.5 ± 0.2%) and (PG: 8.75 ± 0.9% vs 2.5 ± 1.45%)]. The results showed a tendency (P = 0.067) of greater reductions in PMNs in SFPG and CPG treatments, and smaller in PG. The P/AI in the first, second and third cycle after treatment was to SFPG: 45.4%, 33% and 33%, CPG: 30%, 14.3%, and 33.3% and for PG: 25%, 14.3%, and 33.3%, respectively. After three cycles the accumulated P/IA was 72.7%, 60%, and 55.5% (P = 0.769). The treatments reduced PMN with satisfactory rates of P/AI. The continuity of the study with a larger number of animals is required to confirm the indications of these findings.

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A249 Embryology, Developmental Biology and Physiology of Reproduction

Post puerperal endometritis treatment in dairy cows

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Keywords: cefapirin, endometritis, uterine infusion.

This study was aimed to evaluate and compare the efficacy of Cefapirin (Metricure®; MSD; São Paulo-SP; Brazil) with other conventional antimicrobial applied in local route, for the treatment of post puerperal infections. The experiment was accomplished for a sample of 90 cows between the 3rd and the 5th week postpartum, divided into three groups according to birth order. This experiment was approved by the ethics committee of the University of José do Rosário Vellano with the protocol number 0024/2013. The first group (n = 30) received a single uterine infusion with 500 mg Cefapirin, the second (n = 30) was treated with infusion of 2 g of oxytetracycline hydrochloride diluted in 50 mL of saline (single dose) and third (n = 30) received only uterine infusion with 20 mL of saline (single dose). The total rate of clinical cure was 73.3% for Group 1, 46.7% for group 2 and 10% for Group 3 ($P < 0.05$). In relation to uterine cytology results of groups 1 and 2 were compared, and it is considered negative cytology animals with less than 10% of polymorphonucleate cells. Then, the first group was 81.8% and the second group with 57.1% negative cytology ($P < 0.05$). Regarding the reproductive performance after treatments the service period (SP) of the group 1 was 142.3 days and Group 2 164.5 days ($P < 0.05$). In addition, the Group 1 also obtained the highest number of pregnant animals to 1st insemination and 60, 90, 120 and 180 days postpartum. Therefore, it was concluded that the uterine infusion with Cephapirin 500 mg in single dose for the treatment of endometritis showed higher efficiency than conventional treatment with Oxytetracycline.

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A250 Embryology, Developmental Biology and Physiology of Reproduction

Use of different intravaginal devices and doses of progesterone to induce synchronous estrus in Santa Inês sheep

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Keywords: embryo transfer, induction of estrus, sheep

The embryo transfer (ET) routine in sheep implies in successive and increased use of gonadotropin stimuli to ewes. Because these conditions can decrease the fertility of the females, it has been recommended to interval ET procedures with gestations. The objective of this study was to test the efficiency of protocols to induce synchronous estrus in Santa Inês donor ewes. The study was conducted from January to March in Coronel Pacheco-MG. Twenty four ewes previously subjected to six successive non-surgical embryo recovery were equally allocated according body weight (kg) and condition score (BCS; variation 1 to 5) in three experimental groups. Ewes of G1 (n=8; 57.6±12.0 kg; 3.3±0.8), G2 (n=8; 58.5±13.8 kg; 3.4±0.5) and G3 (n=8; 56.6±13.5 kg; 3.3±0.6) received intravaginal devices containing P4 for six days plus 37.5µg d-cloprostenol (Prolise®; ARSA S.R.L., Buenos Aires, Argentina) latero-vulvar and 300 IU eCG (Novormon 5000®; Coopers, São Paulo, Brazil) i.m. 24 h before device removal. It was used CIDR (G1; CIDR®; 330mg P4, Pfizer Saúde Animal, São Paulo, Brasil), human absorbent (G2 and G3; O.B®; Johnson & Johnson, São José dos Campos, Brazil) imbibed with 200 (G2) or 400mg (G3) P4 (Evocanil®; Zodiac Produtos Farmacêuticos, Diadema, Brazil). After device removal, ewes were twice daily (morning/afternoon) checked for estrus and natural mated not exceeding four mating per ram per day.Statistical data are presented in descriptive form. Estrous response and interval to estrus were respectively 100.0% and 41.3±12.2h for G1, 62.5% and 30.0±7.0h for G2 and 100.0% and 28.5±14.2h for G3. Pregnancy rate was 50% (4/8), 25% (2/8) and 50% (4/8) to ewes from G1, G2 and G3, respectively. Overall pregnancy rate considering only ewes mated was 47.6% (10/21). Results of this study suggest that the use of alternative device and source of P4 can be considered to induction of synchronous estrus in Santa Inês ewes after successive non-surgical embryo collections.

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A251 Embryology, Developmental Biology and Physiology of Reproduction

Progesterone concentrations as marker of monitoring equine placental changes and fetal viability

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Keywords: mares, pregnancy, progesterone.

Placentitis is the most common cause of abortion and stillbirth in the horse, compromising fetal-maternal unit by hypoxemia or infection (McKinnon; AAEP; 11; 87; 2009). The aim of this study was to evaluate the progesterone (P4) as a potential marker of monitoring placental changes and equine fetal viability. For this study were used 10 mares with induced ascending placentitis, according to the method already described (Feijó; Arq. Bra. Med. Vet. Zootec; 66; 1663; 2014). Five induced mares were untreated and five induced mares were treated with Trimethoprim- Sulfamethoxazole (30mg/Kg, 12 hours, intravenous) and Flunixin Meglumine (1.1mg/Kg, 24 hours, intravenous). The treatment begging 48 hours post induced to foaling or abortion. Serum sample were collected previous induction (15 days), at induction day, post-induction (2 to 6 days) and at parturition to quantification of progesterone by Immunotech® (Beckman Coulter Company, Marseille, France) comercial diagnosis radioimmunooassay technique. All foaling was assisted and foal viability were evaluated and classified as: A) viable and B) stillbirths or debilitated do not survive. All viable foals were monitored every day by 30 days of age. It was performed grossly and histopathologic evaluation of placentas. The mares were divided into two groups, according histopathological changes in placenta: 1) subacute and acute placentitis; 2) chronic placentitis or unchanged. Groups of placental histology and placentitis treatment were compared by t test. To compare the viability of foals was used kruskal-wallis test, significance was assigned to all values $P<0.05$. No difference was observed in the P4 concentrations at pre-induction, inducton day, post-induction and at parturition in relation to placental injury and treatment. In the post-induction evaluation, mares that delivered viable foals showed higher P4 concentration ($P=0.034$) in relation to the stillbirth foals or debilitated do not survive, the results of mean and standard deviation are respectively: 23.48±20.10 and 8.90±3.47 ng/mL. At birth, P4 did not differ regarding the viability of the foal. All dead or unviable foals (group B) came from mares with subacute or acute placentitis (group1). Progesterone not proved to be a good marker of placental alterations. The progesterone values are lower in mares foaling stillbirth foals or debilitated that do not survive, suggesting that this may be used as an indicator of fetal/neonatal viability.

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A252E Embryology, Developmental Biology and Physiology of Reproduction

A novel 3-D culture system to study bovine oviduct physiology, gamete interaction and early embryo development

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Keywords: 3-D culture system, cattle, embryo development, gamete interaction, oviduct physiology.

Successful fertilization depends on processes that take place in the oviduct. Due to its intra-abdominal location, it is difficult to study intra-oviductal processes *in vivo* in mammals. Instead, *in vitro* models that retain essential cell morphological and functional characteristics are being developed. In culture, bovine oviduct epithelial cells (BOECs) rapidly lose differentiated cell properties (e.g. secretory activity and cilia), while suspended cells have a limited lifespan. Progress with insert culture models and 3-D printing technologies prompted us to develop two independent BOEC culture systems, in which *in vivo*-like differentiation and function is re-established, to study oviduct physiology: (i) 3-D printed U-shaped inserts mounted with PET membranes with 0.4 µm pores (3D U-shaped culture) and (ii) hanging inserts (polycarbonate with 0.4 µm pores) containing 150 µL of Matrigel (3D culture). BOECs were harvested by scraping, and cultured for 24h to agglomerate into floating vesicles with outwardly oriented cilia. The vesicles were plated and, 7 days later, the resulting monolayers were scraped, washed and seeded onto the 2 systems described above and cultured at an air-liquid interface. For comparison, BOECs were also seeded onto coverslips as monolayers (2D culture). After 28 days, the apical side of all BOEC monolayers was washed to harvest secreted proteins, and the inserts were fixed for immunocytochemistry. Proteins (20 µg) were separated by SDS-PAGE and visualized by silver staining, or blotted onto nitrocellulose and immunostained for oviduct specific glycoprotein (OVGP1). Epithelial cell differentiation was indicated by immunodetection of laminin and the presence of primary cilia. Ciliated cell presence (acetylated α-tubulin) and secretory activity (OVGP1) characteristics of BOECs in 3D cultures were comparable to freshly harvested BOECs. The 3D culture yielded 46 silver-stainable protein bands versus 30 in 2D cultures (n=3 per system). In 3D U-shaped cultures, the polarized state (laminin and primary cilia) and their amenability to direct fluorescence microscopy (allowing live cell imaging) are currently determined. In conclusion, 3D culture methods promote polarization and differentiation of BOECs. The extent to which physiological function is maintained is under investigation. Studies in progress to assess the BOEC differentiation using the 3D U-shaped cultures include basolateral co-culture of stromal cells. Ultimately, we aim to develop an oviduct-like environment to study gamete activation, fertilization and early embryo development *in situ*.

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A253E Embryology, Developmental Biology and Physiology of Reproduction

Detection of pregnancy-associated glycoproteins (PAGs) in prolific and non prolific ewes from early to late gestation and postpartum

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Keywords: ELISA, PAGs, non prolific, prolific, RIA, sheep.

Pregnancy-associated glycoproteins (PAGs) are placental antigens that were initially characterized as pregnancy markers in the maternal circulation of bovine species (Zoli et al., 1992, Biol. Reprod. 46: 83-92). After that, the measurement of such molecules in maternal blood as a method for pregnancy diagnosis in other ruminants has been demonstrated in several species. It can give useful information to develop appropriate feeding strategies for pregnant females and to assure requirements of the mother and the growing of fetuses and to avoid metabolic disorder associated to pregnancy. The aim of the present study was to investigate the use of a PAG ELISA-Sandwich kit (Ref. code E.G.7. CER. Marloie, Belgium) *vs* two homologous radio-immunoassay described in El Amiri et al. (2007; Reprod. Domest. Anim. 42:257-62) to detect PAGs in blood samples collected from Boujaâd (non prolific, n=8) and Boujaâd x D'man (prolific, n=20) sheep from early to late gestation and postpartum. Ewes were assumed to be pregnant when PAG concentrations were higher than 0.8 ng/ml in ELISA and 0.3 in RIAs. In addition the samples were also explored by the double immunodiffusion radial (El Amiri et al., 2003, Theriogenology. 59:1291-301) after PAG extractions. The results show that in both systems (ELISA *vs* RIAs), the PAG concentrations were significantly lower in Boujaâd a non prolific sheep than in Boujaâd x D'man a prolific sheep. Furtemore, the concentrations in RIAs were 3 folds higher than those in ELISA. In all systems, the concentrations decreased rapidly after lambing (21 weeks) reaching basal values at fourth week postpartum in ELISA *vs* RIAs. In ELISA all pregnant females showed PAGs level above 1.4 ng/ml from day 24. The double radial immunodiffusion showed positive reactions in ewes carrying more dead fetus. In conclusion, the plasma PAG investigated in the present study showed that the ELISA technique is proved to be a convenient and reliable means for early pregnancy diagnosis as well as for pregnancy fellow up in sheep. From 24 days of gestation, its reliability achieved 100% and, therefore, matches conventional approaches of pregnancy detection. The PAGs could also be detected after extraction from plasma of pregnant ewes using the double radial immunodiffusion. However, for using this technique in routine, further studies are necessary.

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A254E Embryology, Developmental Biology and Physiology of Reproduction

Effect of asynchronous embryo transfer on glucose transporter expression in equine endometrium

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Keywords: asynchronous embryo transfer, endometrium, expression of the glucose transporter, horse.

Equine pregnancy is characterized by an unusually long pre-implantation period (40 days) during which the conceptus is entirely dependent on uterine secretions for nutrient provision. Moreover, horse embryos tolerate a wide range of uterine asynchrony following embryo transfer (ET); however negative asynchrony (recipient behind the donor) of more than 5 days markedly retards conceptus growth and development, and thereby offers a unique tool for studying the effect of the uterine environment on early development. Glucose is an important nutrient during pre-implantation development, however little is known about its transport from the endometrium into the uterine lumen. The aim of the current study was to evaluate the effect of uterine asynchrony on glucose transporter expression in the equine endometrium. Day 8 horse embryos were transferred to recipient mares that ovulated on the same day (synchronous; n=10), or 5 days after (asynchronous; n=10) the donor mare. The resulting conceptuses and matched endometrial biopsies were collected 6 or 11 days after ET (14 or 19 days of embryo development: n=5 per group). Endometrial expression of mRNA for glucose transporters was evaluated by qRT-PCR, and the effects of asynchronous ET and stage of pregnancy were analyzed by two-way ANOVA followed by independent-samples t-tests. Gene expression for SLC2A3, 4, 5, 8, 10 and SLC5A1 was stable over time and treatment, whereas endometrial SLC2A1 mRNA expression was down-regulated in the asynchronous group at day 14 of embryonic development ($p<0.05$), but did not show differences between the two treatment groups at day 19. In summary, the expression of SLC2A1, one of the main glucose transporters in the endometrium, is negatively affected by asynchronous ET and, although its expression seems to be restored by day 19 of conceptus development, this might be a contributor to the delayed development observed in asynchronous pregnancies.

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A255E Embryology, Developmental Biology and Physiology of Reproduction

The effects of hypo- and hyperglycemia during lipolysis-like conditions on bovine oocyte maturation, subsequent embryo developmental and glucose metabolism

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Keywords: glucose, metabolic disorders, NEFA, oocyte.

Elevated follicular NEFA concentrations, commonly present in cattle in NEB or women suffering obesity or type 2 diabetes, are known to disrupt oocyte and embryo development and alter subsequent embryo metabolism. However, NEB cows exhibit systemic hypoglycemia whereas humans suffering metabolic disorders have hyperglycemic insults. Both metabolic features may affect oocyte development. Little is known about whether elevated NEFA concentrations in combination with hyper- or hypoglycemic conditions influences oocyte viability. In this study, we hypothesized that glucose interacts with high NEFA levels during *in vitro* oocyte maturation to affect developmental capacity and metabolism of the resulting blastocysts. Thus, 647 bovine grade I COCs were matured (3 repeats) under 4 conditions: 1) physiological NEFA (72µM; palmitic, stearic and oleic acid) and routine IVM glucose (GLUC) concentrations (5.50mM) (CNTRL), 2) pathophysiological NEFA (420µM) and routine GLUC (HI NEFA), 3) HI NEFA and high GLUC (10mM) (HI NEFA+HI GLUC) and 4) HI NEFA and low GLUC (2.75mM) (HI NEFA+LO GLUC). Subsequently, matured oocytes were routinely fertilized and cultured for 7 days. At day (D) 7 post insemination (pi) all blastocysts were individually cultured for 24 hours in 4µl drops of modified SOF medium under oil after which droplets were analyzed on GLUC concentrations as described by Guerif *et al.* (PLOSone, 8, e67834, 2013). Cleavage (48h pi), blastocyst rates (D8 pi) and the rates of D8 blastocysts from cleaved zygotes were recorded. Developmental competence and GLUC consumption data were compared between 4 treatments using a binary logistic regression model and mixed model ANOVA, respectively. Replicate, treatment and the interaction of both factors were taken into account (IBM SPSS Statistics 20). Significant lower cleavage rates were observed for HI NEFA+LO GLUC (56%) compared with CNTRL (73%; P=0.006) and HI NEFA+HI GLUC conditions (70%; P=0.048). At D8 pi, blastocyst rates of HI NEFA+LO GLUC exposed oocytes (18%) were significantly lower compared with CNTRL (38%, P<0.001), whereas development of HI NEFA+HI GLUC D8 blastocysts (25%) tended to be reduced compared with CNTRL (P=0.066). The capacity of cleaved zygotes to develop to blastocyst stage by D8 showed a similar profile: HI NEFA+LO GLUC (32%) significantly reduced and HI NEFA+HI GLUC (35%) tended to reduce development compared with CNTRL (53%; P=0.024 and P=0.066, respectively). Interestingly, with no significant difference in developmental stage at D7, these HI NEFA+LO GLUC blastocysts consumed significantly less GLUC from D7 to D8 (12.14 ± 4.10 pmol/embryo/h) compared with CNTRL (25.53 ± 2.96 pmol/embryo/h; P=0.020). In conclusion, low GLUC concentrations seem to be more deleterious than high GLUC concentrations in the presence of elevated NEFAs in terms of embryo development and the lower ability of the surviving D7 embryo to consume GLUC as an energy source for its further development.

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A256E Embryology, Developmental Biology and Physiology of Reproduction

Integreated andrological evaluation in Angora goat

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Keywords: integrated andrological evaluation, sperm parameters, testicular parameters.

Integrated andrological evaluation (IAE) is a practice to identify Satisfactory (S), Questionable (Q) and Unsatisfactory (US) males. IAE procedure mainly involves classical and modern methods. The routine evaluation system contains physical and reproductive examination, while the innovative approach is more based on ultrasound detection of testicular and accessory glands, scrotal surface thermography, GnRH challenge test, CASA semen analysis (Computer Assisted Semen Analyzer), fluorescent staining, seminal plasma biochemistry, testicular fine needle aspiration cytology (TFNAC). The aim of this trial was to conduct a pilot study with an IAE based evaluation system (except of the TFNAC) in four healthy adult (4/6 years) Angora goat in Kazan-Turkey. Physical traits such as age, BSC, hereditable defects on: eyes, mouth, legs/feet, prepuce, penis, scrotum and its components were recorded and scrotal circumference, testicular ultrasound (ESAOTE MyLab5, Genoa, Italy with convex probe 2.2 – 6.6 MHz) and scrotal thermography (Flir, E60 during GnRH challenge test with 8.4 μg Busereline IV) were performed. Semen parameters such as color, volume, concentration, sperm motility (SCA Microptics), viability and morphology (Eosin-Nigrosin stain), acrosome integrity (FITC-PNA) were measured in fresh and frozen-thawed semen samples. Correlation indices and mathematical tendencies were calculated using Sigma Stat Software 2.05 and Microsoft Excel version 14.4.9. Three males were evaluated as Q, and one as US because of the presence of feet and mouth defects. One buck has not been evaluated by reasons of higher delta Testosteronemia during GnRH Challenge Test and echotexture testicular classification (Lower Mineralization Index). All mature bucks showed similar scrotal thermal pattern. Seminal plasma mean values of cholesterol, glucose, LDH, triglycerides, total protein, GGT and magnesium were 30.5 mg/dl, 77.8 mg/dl, 470.1 u/L, 8.8 mg/dl, 82.5 g/l, 46.8 u/L and 2.03 mg/dl, respectively. Bucks with higher testicular functionality, according to the physical examination, had the best freezability (Delta Viability and Intact Acrosome) and higher levels of cholesterol (34.5 mg/dl) glucose (87.4 mg/dl), LDH (585.4 u/L), triglycerides (11.15 mg/dl), total protein (89.0 g/l), GGT (53.4 u/L) as well as the lower levels of magnesium (1.88 mg/dl) in the seminal plasma. A correlation between testicular functionality and frozen-thawed semen parameters was also confirmed by sperm kinetic parameters, viability and morphology results. Application of IAE in Angora goat may indicate the buck selection for specific purposes such as breeding, cryopreservation or exclusion from any application.

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A257E Embryology, Developmental Biology and Physiology of Reproduction

Effect of epidermal growth factor on nuclear and cytoplasmic *in vitro* maturation of guinea pig oocytes

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Keywords: apoptosis, cortical granule, EGF, guinea pig, mitochondria, oocyte maturation.

The guinea pig may represent an animal model for research on ovarian infertility and improvement of the in vitro maturation (IVM) conditions is needed in this species. The aim of the present work was to immunolocalize the Epidermal Growth Factor (EGF)-Receptor in the guinea pig ovaries and to study the effect of EGF on meiotic and cytoplasmic maturation, and apoptotic rate in cumulus-oocyte-complexes (COCs). Immunohistochemistry was performed in paraffined ovaries using a rabbit polyclonal antibody EGF-R (1:100; Santa Cruz Biotechnology) and the ABC Vector Elite kit (Vector Laboratories). For the IVM, COCs were collected by aspiration of follicles >700µm under a stereoscopic microscope. They were cultured at 37°C in 5% CO2 during 17 h with TCM-199 supplemented with glutamine, pyruvate, BSA, and different concentrations of EGF (Sigma) [0 (control), 10, 50 or 100 ng/mL] or 10% Fetal Calf Serum (FCS). After IVM, 564 oocytes were fixed and stained with 10 µg/mL Hoechst to assess nuclear configuration in terms of Metaphase II (MII) rate. A total of 143 oocytes were treated progressively with 0.5% pronase, 4% paraformaldehyde, 0.02% Triton X-100, 7.5% BSA and 100 µg/mL FITC-LCA for cortical granule (CG) staining. Also, 78 oocytes were stained with 180 nm MitoTracker RedCMXRos (Molecular Probes Inc) for active mitochondria visualization. CG and mitochondria patterns were analyzed with laser scanning confocal microscopy (Leica TCS SP2). Apoptosis rate in cumulus cells (n=58 COCs) were visualized with TUNEL (In Situ Cell Death Detection Kit, Roche) and analyzed with Image J software. Chi-square test was used to compare nuclear maturation, CG and mitochondria migration rates. The apoptotic index was analyzed by a one-way ANOVA using Duncan post-hoc test. Positive immunostaining for EGF-R was found in granulosa and theca cells and oocytes in all follicular stages. MII were significantly higher in oocytes supplemented with 50 ng/mL EGF group (75.9%) compared to other experimental groups (43.5, 51.8, 53.7 and 59.5% for 0, 10, 100 ng/mL EGF and 10% FCS, respectively, $P<0.05$). Group matured with 50 ng/mL EGF showed higher rate of oocytes with peripheral migration pattern of CG (compatible with cytoplasmic maturation) compared to control group (71.9 vs. 32.4%; $P<0.05$) and migrated mitochondrial pattern compared to the control group and the group supplemented with 100 ng/mL EGF (80.0% vs. 27.8% and 31.3%, respectively; $P<0.05$). Apoptotic rate was lower in 50 ng/mL EGF (17.2±0.9%) and 10% FCS (16.0±1.2%) groups related to the control one (28.7±1.4%) ($P<0.05$). In conclusion, the presence of EGF-R in guinea pig ovaries, suggests that EGF may exert a direct effect on ovarian function. A dose of 50 ng/mL EGF seems to be the most appropriate concentration for IVM of guinea pig oocytes, since it improves nuclear and cytoplasmic oocyte maturation and reduces apoptosis in the cumulus cells.

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A258E Embryology, Developmental Biology and Physiology of Reproduction

Immunoradiometric assay (IRMA) of Pregnancy-Associated Glycoproteins (PAG) in bovine milk: determination of profiles in ongoing and failed pregnancies

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Keywords: cattle, milk, pregnancy, pregnancy-associated glycoproteins,

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Pregnancy-Associated Glycoproteins (PAGs) are used since early eighties as pregnancy markers in cattle and other ruminant species. Until now, they are mainly assayed in plasma or serum samples by using radioimmunoassay or ELISA systems. In cattle, concentrations of PAG are detectable in maternal blood from Day 28 to Day 30 after fertilization. Milk concentrations are 20-30 times lower than in blood samples and cannot be quantified by existing immunoassay systems. Recently, a new sensitive and robust immunoradiometric assay (IRMA) was developed allowing PAG quantification in bovine milk. Purified bovine PAG 67kDa was used as standard at concentrations ranging from 100 to 50,000 pg/mL. Highly purified immunoglobulins (hp-Ig) were obtained from two distinct rabbit polyclonal antisera by using a specific affinity chromatography (anti-PAG 4B-Sepharose gel). The hp-Ig708 (purified from polyclonal antiserum raised against caprine PAG 55kDa+59kDa) was used as capture antibody (0.01 µg/tube). The hp-Ig727 (purified from polyclonal antiserum raised against purified boPAG67kDa) was used as detection antibody (1:8,000). Radiolabeled streptavidin (125I-Strep; 50,000 cpm/100 µL) was used to reveal the Ab-Ag-Ab-Biot complexes. The aim of this study was to quantify PAG concentrations in bovine milk for pregnancy follow-up in cattle. Milk was collected from pregnant cows (n=20) during the whole duration of lactation until dry-off. Samples were frozen until assay. Before analysis, milk samples were thawed at 37°C, centrifuged (2,500 x g) and fat was removed. Samples giving high PAG concentrations were serially diluted in order to fit with standard curve range. In non-pregnant cows, concentrations remain lower than 40-50 pg/mL at all time points. In pregnant cows, milk PAG concentrations increased from Week 10 (56.9 ± 13.1 pg/mL) to Week 11 (93.5 ± 20.4 pg/mL) and Week 12 (135.2 ± 27.7 pg/mL). Thereafter, PAG concentrations increased regularly until Week 32 (2,177.6 ± 496.2 pg/mL) and slightly decreased until dry-off at Week 35 (1,615.9 ± 663.9 pg/mL). Immediately after parturition, PAG concentrations reached 5,615.3 ± 615.7 pg/mL and decreased continuously until Week 11 postpartum (36.6 ± 2.1 pg/mL). In this experiment, we could also follow three cows with pregnancy failure (2330, 7722 and 7725). Two of these cows (7722 and 7725) showed very low levels of PAG before pregnancy failure. In Cow 2330, PAG concentrations clearly decreased around the time of pregnancy failure. In conclusion, in the present study we describe the use of a sensitive and quantitative IRMA allowing pregnancy follow-up in dairy cows. This approach offers the possibility (in time or in retrospective studies) of an individual follow-up without any additional manipulation of female neither any stress induced by the investigator.

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A259E Embryology, Developmental Biology and Physiology of Reproduction

Cumulus cells protect the oocyte against free fatty acids

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Keyword: bovine cumulus cell, Carnitine-PalmitoylTransferase-1A, DiGlyceride-AcylTransferase, free fatty acids, Stearoyl-CoA-Desaturase.

Cumulus cells have an intimate contact with, and provide metabolites to, the oocyte. The importance of cumulus cells for the oocyte extends into potential protection of the oocyte against free fatty acids (FFA)[1]. Exposure of cumulus-oocyte-complexes (COCs) to elevated FFA levels results in massive lipid accumulation in cumulus cells and normal developmental competence of oocytes (Aardema et al., BoR, 2013; 88, 164). Two potential mechanistic routes by which cells are protected against saturated FFA are lipid storage and β-oxidation (Henique et al., JBC, 2010; 285, 36818-827). To further unravel the presumed protection against FFA by cumulus cells, oocytes with and without cumulus cells were exposed to FFA. To investigate the potential mechanism by which cumulus cells may protect the oocyte, gene expression of cumulus cells from COCs matured in the presence or absence of FFA was analysed for DiGlyceride-AcylTransferase (*DGAT*; lipid storage), Carnitine-PalmitoylTransferase-1A (*CPT-1A*; β-oxidation) and Stearoyl-CoA-Desaturase *(SCD)*, the enzyme that converts saturated FFA into unsaturated. COCs were collected from bovine slaughterhouse ovaries and during 23h matured with or without 250μM saturated stearic acid followed by standard fertilization and culture. After 8h of maturation, cumulus cells were removed from part of the COCs and oocytes were placed back in maturation medium. Gene expression of cumulus cells from COCs was analysed by QPCR for *DGAT, CPT-1A* and *SCD* before and after 23h culture with or without FFA, and from cumulus cells without an oocyte for *CPT-1A* and *SCD*. Statistical analysis was performed by a paired sample t-test (gene expression) and general linear model (culture data). Materials and methods according to Aardema et al. (BoR, 2013; 88, 164) Removal of cumulus cells after 8h maturation resulted in oocytes with normal developmental competence (27±2.8%; 24±1.1% for COCs). Exposure to stearic acid resulted in strongly reduced developmental competence of oocytes cultured without cumulus during the last 15h (1±1.0%; $P<0.01$) compared to oocytes matured as COC (18±4.2%). Expression of *CPT-1A* ($P<0.01$) and *SCD* ($P<0.01$) in cumulus cells increased during maturation of COCs, independent of the presence of FFA. *DGAT* expression was not different among groups. The presence of an oocyte during culture resulted in higher *SCD* expression levels in cumulus cells after 23h of culture ($P<0.05$). These data indicate that cumulus cells are essential to protect the oocyte against saturated stearic acid. The increase in *CPT-1A* expression was independent of the condition and is in line with the necessity of β-oxidation during COC maturation. *SCD* expression has to our knowledge, not been investigated before and showed a marked, oocyte dependent, increase during maturation. We suggest that conversion of saturated FFA into harmless unsaturated FFA by cumulus cells protects the developmental competence of the oocyte.

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A260E Embryology, Developmental Biology and Physiology of Reproduction

Mobilization of intracellular lipids by supplementation of IVM and IVC media with L-carnitine improves bovine embryo quality

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Keywords: gene expression, *in vitro*, mitochondria.

Mobilization of embryo lipid by supplementing culture media with metabolic activator is one of the promising tools to improve quality of in vitro produced bovine embryos. Therefore, the present study investigated the effect of L-carnitine supplementation during in vitro maturation (2.5 mM) and embryo culture (1.5 mM) on embryo developmental rates, quality and gene expression profiles. Cumulus-oocyte complexes recovered from slaughter house ovaries were morphologically evaluated and only grades 1and 2 were used in this study. Treatment groups were: T1=IVM+LC, T2=IVC+LC, T3=(IVM and IVC)+LC and control. In vivo produced embryos were included in all analyses. Development rate was calculated based on the number of embryos reached blastocyst stage at day 8 of culture. Total cell count as well as number of apoptotic cells was evaluated using Tunnel-Hoechst assay. The activity of mitochondria and intensity of lipid was measured using fluorescent probes. Expression of embryo selected candidate genes was profiled using quantitative real-time PCR. Our results showed no differences ($P < 0.05$) in cleavage rate between L-carnitine treated groups and control. Although there was an increase in blastocyst rate in T2 (44.4%) and T3 (42.1%) groups compared to T1 (39.2%) and control (38.2%), it was not statistically significant. Embryos cultured with L-carnitine and in vivo group had greater total cell number (T1: n=140.2, T2: n=164.8, T3: n=155.9 and in vivo: n=160) than the control (n=129.4). On the other hand, the percentage of apoptotic cells from total number of cells was greater ($P < 0.05$) in control (11.2%) than L-carnitine treated (T1: 4.2, T2: 3.8 and T3: 2.9%) and in vivo derived blastocysts (0.3%). Cytoplasmic lipid content was reduced by 1.8, 2.7, 2.4 and 5.1 times in T1, T2, T3 and in vivo produced blastocysts compared to their control counterparts. Whereas, intracellular mitochondria density was increased by 2.0, 4.8, 4.5 and 6.3 folds in embryos cultured with L-carnitine and in vivo group. Genes regulating lipid oxidation (CPT2 and CPT1B), fatty acid transport (SLC27A1) and mitochondria transcription (TFAM) were up-regulated while a lipid storage marker transcript (PLIN2) was down-regulated in embryos cultured in presence of L-carnitine and in vivo ones compared to control. Collectively, the lipolytic effect of L-carnitine was linked with increased mitochondrial activity, reducing apoptotic cells and modulating gene expression of in vitro produced embryos which will most likely enhance their survival after cryopreservation and transfer to recipients.

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A261E Embryology, Developmental Biology and Physiology of Reproduction

Intrauterine expression of insulin-like-growth factor family members during early equine pregnancy

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Keywords: conceptus, horse, IGF-family members, pre-implantation period.

Insulin-like growth factor (IGF) family members are known to regulate fetal and placental growth and development. Insulin (INS), IGF1 and IGF2 stimulate cell proliferation and differentiation via their receptors INSR, IGF1R and IGF2R. The actions of IGF are further regulated by the IGF-binding proteins. The horse is unique with regard to an unusually long pre-implantation period (40 days) offering a unique tool to study the dialogue between conceptus and endometrium. We evaluated the expression of IGF system components in equine conceptus membranes, and endometrium during the cycle and early pregnancy. Endometrial biopsies were harvested on days 7, 14, 21 and 28 from pregnant mares, following conceptus collection, and days 7, 14 and 21 from cycling mares (n=4 per group). Bilaminar trophoblast was isolated from day 14 and 21 conceptuses, and the yolk-sac and allantochorion from 28 day conceptuses were separated. Expression of mRNA for IGF system components (INS, INSR, IGF1, IGF1R, IGF2, IGF2R) were investigated by qRT-PCR. The effect of conceptus developmental stage was analyzed by one-way ANOVA, and the effects of pregnancy and days after ovulation on endometrium by two-way ANOVA followed by independent-sample T-tests. INS mRNA was not detected in endometrium or conceptus membranes. IGF1 and IGF2R mRNA levels were uniform in cycling and pregnant mare endometrium. INSR gene expression increased in the endometrium of pregnant mares only from day 7 to 14 ($p<0.05$) and showed a higher expression than in cyclic mares on day 21 of pregnancy ($p<0.05$). IGF2 mRNA increased sequentially from day 7 to 14 to 21 of pregnancy ($p<0.05$). IGF1R expression was elevated on day 14 in both cyclic and pregnant mares ($p<0.05$). In the conceptus membranes, mRNA expression for INSR, IGF1, IGF1R, IGF2 and IGF2R was low on days 7 and 14 but showed up-regulation from day 21 ($p<0.05$). In summary, IGF family members are expressed uniformly in endometrium from cycling mares whereas endometrial expression increases during early pregnancy. Conceptus membrane expressions of IGF family genes increases from day 21, when the blastocyst capsule would start to disintegrate. We propose that the INS/IGF system plays an important role in early equine embryonic growth and the preparation for placentation.

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A262E Embryology, Developmental Biology and Physiology of Reproduction

Bovine oviduct epithelial cells: an *in vitro* model to study early embryo-maternal communication

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Keywords: bovine oviduct epithelial cells, embryo-maternal communication, IVP.

We aimed in this study to: (1) assess the expression of oviduct epithelial cells markers on bovine oviduct epithelial cells (BOECs) cultured *in vitro* under two different systems (suspension or monolayer) and (2) determine the BOECs response to the presence of early bovine embryos. BOECs were mechanically extracted by squeezing the isthmus regions of oviducts collected from slaughtered heifers during the early luteal phase, determined by the appearance of the corpus luteum. Part of the oviduct extract was frozen in liquid nitrogen and stored at -80^{o}C for gene expression analysis (fresh BOEC), while the rest was cultured in SOF+10% FCS for either 24 h (suspension) or 7 days (monolayer). Suspension or monolayer BOECs were co-incubated for 24 h with Day 2 (2- to 4-cell) or 3 (8- to 16-cell) bovine embryos produced *in vitro* to determine the embryonic effect on BOECs. A control group without embryos was included for each BOEC culture. RNA extraction from BOECs was carried out by Trisure™ (Bioline, Madrid, Spain) and Dynabeads (Dynal Biotech, Oslo, Norway) and gene expression was analyzed by qPCR, using *ACTG1* as housekeeping gene. Statistical differences were assessed by ANOVA. *OVGP1*, *GPX4* and *FOXJ1* were chosen as markers for oviductal epithelial cells and based on their function to support early embryo development, protect gametes against oxidative stress, and cilia formation, respectively. *KERA* and *PRELP* are genes implicated in extracellular matrix and *ROCK2* and *SOCS3* are genes involved in cytokinesis, all of which were found to display a response to the early embryo *in vivo* (Maillo et al., Biol Reprod 2015, DOI:10.1095/biolreprod.115.127969). Among BOECs markers, *OVGP1* and *FOXJ1* were significantly downregulated in suspension cells compared with fresh BOECs, losing their expression in a monolayer; however, *GPX4* was significantly higher in monolayer than fresh and suspension BOECs, suggesting that although monolayer BOECs lost some of their functional characteristics, they still conserved others like protection against oxidative stress. Regarding the effect of the embryos on *in vitro* cultured BOECs, only suspension BOECs showed an embryonic effect on gene expression: *ROCK2* and *SOCS3* were significantly upregulated in cells co-cultured with Day 2 compared with Day 3 embryos. In conclusion, based on the markers studied, BOECs cultured *in vitro* lost some of their functional characteristics, with suspension cells being closer to *in vivo* controls than monolayer. In addition, under our experimental conditions, suspension cells were more adequate to detect possible embryo signals than monolayer.

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A263E Embryology, Developmental Biology and Physiology of Reproduction

Effect of high hydrostatic pressure (HHP) stress on intercellular ATP content in pig embryo

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Keyword: embryo development, hydrostatic pressure, pig.

Embryos exposed to high hydrostatic pressure (HHP) have a greater resistance to further stress and a higher survival rate in cryopreservation or nuclear transfer processes (Pribenszky C., Biology of Reproduction 83; 690-697, 2010). It is known that efficient metabolism is one of the main factors response for a proper development of pig embryos (Romek M., Reproduction in Domestic Animals 46; 471-80, 2011). In addition, preliminary measurements of the inner mitochondrial membrane potential (ΔΨm) has shown lower ΔΨm in HHP embryos compared to untreated embryos. If the HHP directly affects on metabolism of embryos reducing the ΔΨm, perhaps the total amount of adenosine triphosphate (ATP) content is changing. The aim of this study was to examine the effect of HHP treatment of porcine zygote, on ATP level in embryos at various stages of development. Pig zygotes (number of 217 embryos) used in the experiment were collected surgically from superovulated gilts breed polskiej zwisłouchej. Gilts were superovulated by an intramuscular injection of 1500 IU of PMSG (pregnant mare serum gonadotrophin, Serogonadotropin, Biowet) followed 72 h later by 1000 IU of hCG (human chorionic gonadotropin, Chorulon, Biowet). Embryos were treated by HHP in HHP device (Cryo-Innovation Ltd, Hungary) for 1 h in 39°C at a pressure of 20 MPa. Afterwards cultured in vitro in medium NCSU-23 in 39°C and 5% CO2. Before ATP analysis, embryos from experimental and control groups were frozen in 5µl Gibco® HEPES buffer (Thermo Fisher Scientific Inc., MA USA) in 1.5ml eppendorf (4-8 embryos in each tube). Analysis of ATP content was performed using Adenosine 5'-triphosphate (ATP) bioluminescent somatic cell assay kit (Sigma Chemical Company, USA) and the luminometer Lumat^3 LB 9508 (Berthold Technologies, USA). In order to examine the statistical differences a one-way ANOVA were used. The intracellular ATP content in HHP treated group (A) and control group (B) at zygote stage (a), 8-16 cells (b), morula (c) and blastocyst (d), looks like this: Aa 1.63 ± 0.26 pmol/embryo, Ab 1.55 ± 0.25 pmol/embryo, Ac 0.97 ± 0.09 pmol/embryo, Ad 0.88 ± 0.23 pmol/embryo, Ba 1.51 ± 0.40 pmol/embryo, Bb 1.40 ± 0.12 pmol/embryo, Bc 1.01 ± 0.35 pmol/embryo, Bd 0.62 ± 0.15 pmol/embryo. Pig embryos treated by HHP at zygote stage show not significant differences in intercellular ATP content compared to control group. Significant differences in ATP content between zygote, 8-16 cells and morula, blastocyst stages in both groups of HHP treated and untreated embryos were observed. It means that ATP content in pig embryo is changing during development.

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A264E Embryology, Developmental Biology and Physiology of Reproduction

Lipid profile analysis of bovine *in vitro* blastocysts deriving from insulin treated oocytes by desorption electrospray ionization – mass spectrometry (DESI-MS)

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Keywords: blastocyst, cattle, insulin, IVP, lipid profile analysis.

The aim of this study was to characterize the lipid profile of bovine blastocysts produced from oocytes exposed to different insulin concentrations during maturation by DESI-MS. Insulin is a key metabolic hormone and its concentration in blood and follicular fluid changes in situations of metabolic imbalance as obesity, diabetes or negative energy balance (NEB). The impact of insulin on the lipid profile of blastocysts can provide important insights on the metabolic changes induced by this hormone on early development. Blastocysts were produced from abattoir derived oocytes according to standardized IVP-protocols in our laboratory. Insulin treatment was performed during 22 h of maturation using 0 (INS0); 0.1 (INS0.1) or 10 (INS10) µg/ml bovine insulin. After maturation, all treatment groups were submitted to equal conditions during fertilization and culture. On day 8, blastocysts were separately frozen at -80ºC in PBS with 0.1% PVA and individually transferred to glass sides in randomized order. A total of 63 blastocysts were used for DESI-MS lipid profile analysis. Lipids such as diacylglycerols (DAG), triacylglycerols (TAG) cholesteryl esters (CE), squalene and ubiquinone were detected in positive ion mode as silver adducts. Average full scan mass spectra of the three different treatment groups indicated few changes in the lipid profiles. Multivariate statistics by PCA (Principal Component Analysis) was used to comprehensively explore the chemical information of the full mass spectral dataset and visualize the grouping of samples resulting from chemical similarity. PCA showed some extent of discrimination between INS0 and INS10 whereas the discrimination between INS0 and INS0.1 was less evident. Data suggests down-regulated mitochondrial metabolism (indicated by ubiquinone abundance) in INS10 as well as few changes in TAG- and cholesterol metabolism comparing the treated groups (INS10 and INS0.1) with the control (INS0). Overall, the low extension of changes observed in the DESI-MS lipid profiles indicates minimal impact of insulin exposure during oocyte maturation on lipid content during preimplantation embryo development. The results of the lipid profile analysis shows that the lipid profile was not significantly different in the day 8 blastocyst after exposure of insulin during maturation. Possible explanations could be that the insulin exposure during the IVM period is not sufficient to promote extensive end-point metabolism changes in the lipids detected during preimplantation development, or that the early embryo strongly compensates for the impact of a metabolic stressor as insulin during oocyte maturation by a subsequent change in gene expression, leading to compensating mechanisms to obtain balance in the chemical profile and permitting a viable phenotype.

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A265E Embryology, Developmental Biology and Physiology of Reproduction

Laparoendoscopic single site surgery (LESS) approach to the porcine oviduct for *in vivo* evaluation of physicochemical parameter

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Keywords: approach, laparoscopy, monoport, oviduct, pig, single-site surgery.

We aimed to define a surgical approach to the porcine oviduct capable of combining minimally invasive techniques with a time effective and accurate insertion of biosensors of physico-chemical parameters. Gilts (n= 14) and sows (n=6), of a range weight of 85 to 280 Kg were used. Animals were anaesthetized and placed in lateral right recumbent position. Then, a 5-6 cm incision in the skin followed by layer-by-layer surgical approach to the abdominal cavity was done so as to place the single-site monoport device GelPOINT Advanced (Applied Medical®, Rancho Santa Margarita, California, USA). Under laparoscopy conditions -CO2 pneumoperitoneum (8-10 mmHg)- the left uterine horn was grasped with non-traumatic forceps. Pneumoperitoneum and the single port cap were then removed, and the reproductive organs pulled up towards the incision so as to allow a direct manipulation of the oviduct. The rapid identification of the abdominal opening allowed a rapid and effective insertion of biosensors within the lumen, thus allowing the evaluation of the oviduct microambient, i.e. pH, O2 or temperature. After settling and stabilizing the probes within the oviduct lumen, the organs were put back into the abdominal cavity and in vivo recording of physiological parameters started. The laparoendoscopic single-site surgery (LESS) approach was successful in all the animals, independently of weight and reproductive maturity. Manipulation of reproductive organs was always minimal, although in 3 cases (2 gilts and 1 sow), small and slight hyperaemic areas caused by the forceps were observed in the uterine horn. During the approach no damage to the ovary, oviduct or any other abdominal organ such as intestine was produced. The average duration of the whole procedure since the beginning of the incision in the skin till the insertion of the biosensor within the oviduct was approximately 19.5 min (12-27 range), with a current duration of pneumoperitoneum conditions of 5.5 min (4-7 range). Independently of the LESS approach occasional bleeding of the mesosalpingeal vessels was observed during the manipulation required for stabilizing the probes within the oviduct. The laparoendoscopic single-site (LESS) approach described here proved very efficient in terms of allowing a rapid, minimally invasive and hardly manipulative approach to the reproductive organs, and particularly to the oviduct lumen. This approach benefits from the advantages of both laparoendoscopy (minimal trauma) and traditional laparotomy (by-hand manipulation of organs). The LESS approach is been successfully used to evaluate pH, CO2, O2 and temperature within the oviduct.

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A266E Embryology, Developmental Biology and Physiology of Reproduction

Periovulatory pH within the porcine oviduct and uterus obtained by laparoendoscopic single-site surgery

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Keywords: laparoscopy, oviduct, pH, pig, uterus.

To determine *in vivo* pH values within the oviduct (ampulla and isthmus) and uterus in the porcine species with a minimally invasive approach. Eight pre-pubertal gilts (G) and 7 sows (S) were used. G were treated with intramuscular 1500 IU of eCG and 750 IU of hCG to induce ovulation. 36-44 hours after hCG injection (G) or the onset of oestrus external signs (S) pigs were anaesthetized and placed in lateral right recumbent position. A left lateral paralumbar laparoendoscopy single-site surgical approach (GelPOINT Advanced, SingleMedical®) was carried out under CO2 pneumoperitoneum (8-10 mmHg). Laparoscopy non-traumatic forceps were used to pull up the ovary towards the incision and upon visual inspection pigs were assorted into preovulatory (PreO) or postovulatory (PostO) stages. A flexible 1.6 mm diameter pH probe (MI508, Microelectrodes®, New Hampshire, USA) was sequentially inserted into the ampulla (Amp), isthmus (Isth) and uterus (Ut) for a time period of 10-12 min after signal stabilization. A reference electrode (MI401, Microelectrodes®, New Hampshire, USA) was also required for measurements. To simulate the physiological ambient registers were obtained after replacing back the organs -with the pH probe inserted- into the abdominal cavity and the surgical port closed. Anova of repeated measures was carried out with SPSS 19 (IBM®) to evaluate for a significance level of $p<0.05$. pH values (mean ± SD) within the Amp and Isth were significantly different (7.41 ± 0.17 and 7.10 ± 0,21 respectively, $p<0.001$). pH within the uterus (7.55 ± 0.16) was within the range of the Amp ($p>0.05$) and significantly higher than in the Isth ($p<0.001$). Regarding the PreO and PostO stages pH differences were found in the oviduct ($p=0.02$) for either the Amp (7.45 ± 0.15 vs 7.34 ± 0.12) or the Isth (7.15 ± 0.24 vs 7.04 ± 0.12), but not for the Ut (7.57 ± 0.15 vs 7.52 ± 0.07). While no differences between G and S were observed (7.35 ± 0.24 vs 7.33 ± 0.25) a significant interaction between sex maturity (G vs S) and the phase of the estrous cycle (PreO vs PostO) was found ($p=0.02$). The recorded pH values in the oviduct were lower than those of Nichol (Can. J. Physiol. Pharmacol. 75:1069, 1997), which could be related with the use of a different pH probe and surgical approach. The pronounced pH contrast between the Amp and Isth, and between the Isth and Ut is a relevant result that should be considered to better understand the microambient experienced by the porcine gametes and early embryos.

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A267E Embryology, Developmental Biology and Physiology of Reproduction

Omega-3 fatty acids enhance developmental competence of bovine oocytes under metabolic stress conditions *in vitro*

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Keywords: blastocysts, bovine oocytes, developmental competence, IVP, Omega-3 fatty acids.

Metabolic stress conditions such as negative energy balance in dairy cows are associated with fat mobilization and elevated saturated (stearic; SA, palmetic; PA) and monounsaturated (oleic; OA) fatty acids (FAs) in serum and follicular fluid. We have shown that these FAs have direct detrimental effects on oocyte quality (Van Hoeck et al., ARS, 149:19-29, 2014). In contrast, we demonstrated that polyunsaturated α-linolenic acid (*n-3* 18:3; ALA) can enhance oocyte competence (Marei et al., BOR, 81:1064-1072, 2009). Here, we examined the effects of ALA supplementation (at physiological follicular fluid concentration; 50 μM) during *in vitro* oocyte maturation on subsequent embryo development in the presence of high follicular fluid concentrations of SA, PA and OA (HNEFA, 425μM). Cumulus cell expansion was scored at the end of oocyte maturation (0-3: 0; not expanded, 3; fully expanded). The proportions of cleaved and fragmented embryos were recorded on day 2 post-fertilization. Blastocyst rates were recorded on day 7 and 8. Day 8 blastocysts were categorized as Normal (not expanded), Expanded, or Hatched, and were fixed and immunostained with anti-cleaved-caspase-3 antibody and Hoechst. Total cell counts and apoptotic cell indices were calculated. Data were obtained from 5 independent repeats using 1529 oocytes derived from slaughter house material. A total of 179 blastocysts were stained. Categorical data were analyzed by binary logistic regression using SPSS, and numerical data were analyzed using ANOVA. Pairwise comparisons were performed using Bonferroni correction. *P* values <0.05 were considered significant. Compared with FA-free solvent controls, supplementation with HNEFA resulted in: inhibition of cumulus cell expansion (score: 1.7±0.2 vs. 2.8±0.04, $P<0.05$); higher fragmentation rates (16.8% vs. 9.5%, $P<0.05$); and lower blastocyst rates on day 7 ($P<0.05$), either expressed as a proportion from the total number of fertilized oocytes (15.6% vs. 22.8%) or from the total number of cleaved embryos (20.4% vs. 30.6%). Hatched and expanded blastocysts produced from HNEFA-exposed oocytes had higher apoptotic cell indices. In contrast, these negative effects were alleviated by ALA supplementation. In the HNEFA+ALA group, cumulus expansion score (2.4±0.16), fragmentation (6.9%), blastocyst rate on day 7 (21.4% from total fertilized oocytes and 28.7% from cleaved embryos), and apoptotic cell index were similar to the controls. In addition, HNEFA+ALA group had significantly higher total cell numbers in expanded and normal blastocysts compared with those from HNEFA group. In conclusion, ALA supplementation enhanced oocyte developmental capacity during maturation under metabolic stress conditions. The underlying mechanisms of action are currently under investigation. These results may have clinical implications to improve fertility through dietary interventions in animals and humans suffering from metabolic disorders associated with lipolysis.

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A268E Embryology, Developmental Biology and Physiology of Reproduction

Addition of omega-3 DHA during *in vitro* maturation affected embryo development

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Keyword: bovine oocytes, *in vitro* maturation, omega-3 DHA.

Several studies have suggested a positive effect of n-3 poly-unsaturated fatty acids (PUFA) on bovine reproduction. Indeed, n-3 PUFA reduced prostaglandin secretion in uterine environment, thus providing more favorable conditions for embryo development. Other studies suggested a direct effect of n-3 PUFA on the oocyte that could enhance fertility. In the present study, we aimed at investigating *in vitro* the effect of docosahexaenoic acid (DHA, C22:6 n-3, Sigma, Saint-Quentin Fallavier, France) on bovine oocyte maturation and developmental competence. Oocyte cumulus complexes (OCC) were collected from slaughtered cows. In first experiment, *in vitro* maturation (IVM) with DHA 1, 10 and 100 µM was performed (n=3 replicates, 50-60 OCC per condition). After IVM, oocyte viability was assessed using Live/DEAD staining and then meiotic stages were determined by using Hoechst staining after oocyte fixation. Neither difference in viability nor in maturation rate was observed after IVM between control and treated oocytes whatever the DHA concentration. 83.1% of mature oocytes in control IVM and 78.9%; 84.0%; and 84.0% in presence of DHA at 1, 10, 100 µM, respectively, were observed. In second experiment (n=5 replicates, 50-60 OCC per condition), after 26h IVM with or without DHA 1, 10 and 100 µM, oocytes were subjected to parthenogenetic activation (ionomycin 5 µM, 5 min and 6DMAP 2 mM, 4h). Oocytes were then *in vitro* developed in modified synthetic oviduct fluid supplemented with 1% estrus cow serum for 7 days. Cleavage rate and a number of blastomers were assessed in resulting embryos at day 2 post activation. Cleavage rate significantly increased after IVM with DHA 1µM (84.3%) but significantly decreased with 100µM DHA (66.2%) as compared to control (76.0%) embryos (Chi-square test p=0.02). Moreover, the percentage of embryos that progressed further than 4 cells at day 2 was significantly higher (p=0.02) in the presence of 1 and 10 µM DHA (40.8% and 40.4%, respectively) than in control (31.2%) and with DHA 100 µM (22.2%). At day 7, embryos from DHA 1 µM-treated oocytes encountered more cells than those from control and other DHA groups (10 and 100 µM). Altogether these data suggest that a low dose of DHA (1µM) during IVM might improve oocyte developmental competence through possible effect on cytoplasm but not nuclear maturation. Also, we confirmed that a high dose of DHA (100µM) is deleterious for oocyte developmental potential.

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A269E Embryology, Developmental Biology and Physiology of Reproduction

Periconceptional body condition induces placental adaptations but does not affect foal growth and metabolism in horses

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Keywords: body condition, foal, glycemia, horse, periconception, placenta, pregnancy,

Objectives: It has been shown in several species that the periconceptional environment can affect offspring long-term phenotype. This study aims to investigate the effects of periconceptional body condition on feto-placental biometry, post-natal foal growth and glucose metabolism. Materials and methods: 32 saddlebred mares of similar size were allocated to one of two groups depending on their body condition score (BCS, 1-5 French scale) at the time of artificial insemination (AI). Group High (H, n=18) had a median BCS of 3.9 (range: 3–4.25) whereas group Low (L, n=14) had a significantly lower BCS (median: 2.5, range: 2–3.75, p=0.01). Both groups were kept in pasture until the 7th month of gestation when they were housed indoors and fed forage and concentrate (barley). Food intake was not different between groups. Mares were weighed every 2 weeks and their BCS was monitored monthly. Placentas and foals were weighed and measured at birth. Foals were measured and their fasting glucose assessed regularly until 12 months of age. A frequently sampled intravenous glucose tolerance test (FSIGT) was performed at 3 days and 4 months of age. Results were analyzed using a Mann-Whitney test. Results: H mares maintained a significantly higher BCS (median ≥3.75) than L mares from AI until foaling (median at foaling: 3.75, p<0.0001). L mares reached a peak BCS of 3.75 at the 7th and 8th month and thereafter lost BCS until foaling (median BCS at foaling: 2.75). Mares' body weight was not different between groups at any time. Gestation length did not differ between groups. H placentas tended to be 15% lighter with a 10% reduced surface compared to L placentas (p=0.071). Foals' weight and measurements at birth were not different but the placental efficiency (foal/placental weight) tended to be 12% higher in H mares (p=0.078). There was no difference in foals' growth until 12 months. H foals' fasting glucose tended to be higher at 3 days (p=0.063) but there was no difference in the glucose response to the FSIGT. Plasma insulin concentrations are pending. Conclusion: H mares tended to have a lighter placenta and with a reduced surface area that was more efficient than L mares. Their foals tended to have greater fasting plasma glucose than L foals at 3 days. The fact that the BCS of H and L mares throughout gestation matched their BCS at AI highlight the importance of periconceptional BCS. This study follows a previous one showing that feeding mares in the 2nd part of gestation with two different energy sources does not affect feto-placental biometry and foal development until the age of 6 months (Peugnet et al. 2015, Plos One 10, e0122596). Nevertheless, periconceptional BCS appears to induce placental adaptations that are currently being characterized.

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A270E Embryology, Developmental Biology and Physiology of Reproduction

Involvement of phosphodiesterase 5 (PDE5) on lipid accumulation in bovine oocytes and embryos produced *in vitro*

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Keywords: cryotolerance, IVP, lipid metabolism, melatonin, nucleotide.

The aim of this study was to investigate the involvement of PDE5 on lipid metabolism in bovine oocytes by assessing the effects of PDE5 inhibition during in vitro culture on lipid contents in oocytes and resulting in vitro produced (IVP) embryos, and their cryotolerance. In Experiment 1, cumulus-oocye complexes (COCs) from slaughterhouse ovaries were submitted to IVM in TCM199 supplemented with 0.4% BSA or 10% FCS associated or not with a PDE5 inhibitor (10^{-5}M sildenafil- Sigma-Aldrich) and after 22h oocytes were denuded and stained with Nile Red (1µg/ml, 30 min) to assess cytoplasmic lipid levels measured by fluorescence intensity. In Experiment 2, 10^{-5}M sildenafil (SDF) was included during IVM and/or IVC (SOFaa) during embryo development after IVF (TALP medium using frozen sperm from the same bull prepared by Percoll gradient). Controls were cultured without SDF and all groups were cultured with 10% FCS. After 22h IVM, 20h IVF and seven days IVC, embryos were assessed for cleavage (Day 4) and blastocyst development rates. Day 7 blastocysts (BL) were fixed and stained with Nile Red to evaluate lipids. In Experiment 3, the same groups were assessed plus two others including melatonin (10^{-7}M) as an antioxidant during IVC in SDF treated groups. Cleavage and BL rates were determined and embryos were vitrified. After thawing, BLs were cultured for 24h to assess reexpansion and 48-72 h for hatching. Cultures were at 38.5ºC under 5%CO_2 in air. Statistical analyses were performed by ANOVA followed by Tukey test using SAS and significance level was 5%. In Experiment 1, SDF reduced ($P<0.05$) lipid content in oocytes matured with BSA (13.1) or FCS (16.3) when compared to controls matured only with BSA (17.6). SDF groups were similar ($P>0.05$). Reduction in lipids was only observed in BLs produced with SDF in both IVM and IVC (30.2; $P<0.05$). Oocytes matured only with FCS had highest lipid content (20.1, $P<0.05$). In Experiment 2, there was no effect of SDF or melatonin on cleavage or BL rates (79 and 31%, respectively, $P>0.05$) or reexpansion and hatching (89 and 64%, respectively, $P>0.05$). In conclusion, PDE5 inhibition during IVM reduces lipid content in oocytes, but in embryos, inhibition is necessary during both IVM and IVC. Lipid reduction, however, did not translate into improved cryotolerance, neither did the addition of the antioxidant melatonin. PDE5 appears to be involved in lipolysis in bovine oocytes and embryos possibly related to cGMP levels and PKG activity and may be an interesting target for studies to understand lipid metabolism in oocytes and IVP embryos. To our knowledge, this is the first study to show the possible relationship between this pathway and lipid metabolism in bovine oocytes and embryos.

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A271E Embryology, Developmental Biology and Physiology of Reproduction

In vitro production of bovine embryos as a toxicological model: impact of polychlorinated biphenyl (PCB) 126 during maturation

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Keywords: 3R, blastocysts, environmental pollutants, neutral lipids.

Many of the experimental animals used in toxicological studies are for assays involving reproductive toxicity and the vast majority use the small rodents as models for the human. There are many factors making the human and cow much more similar than humans and rodents. The aim of this study was to explore the bovine IVP system for the impact of PCB 126 during oocyte maturation. All PCB congeners are lipophilic persistent environmental pollutants, of which PCB 126 is the most dioxin-like (activates the aryl hydrocarbon receptor) and therefore considered to be the most toxic congener. For maturation, 254 abattoir derived oocytes were used (in three replicates). The oocytes were randomly divided into two groups for maturation and the treated group contained an addition of 100.6 pg/ml of PCB 126, a concentration previously found to affect cleavage and blastocyst development (Krogenæs et al., Reprod Toxicol 12:575-80 1998). Apart from the addition of PCB 126, the maturation, fertilization and culture were done according to standardized protocols (Abraham et al., Acta Vet Scand 54:36 2012). The embryo development was assessed through cleavage at 44 h after fertilization and blastocyst development (stage and grade) at day 7 and 8 after fertilization. At day 8 after fertilization the blastocysts were stained for number of nuclei (DraQ-5, Bionordica, Stockholm, Sweden) and neutral lipid (HCS LipidTOX, Invitrogen, Paisley, UK). The embryos (n = 63) were examined for number of nuclei and for neutral lipid staining intensity with fluorescent microscopy and ImageJ 1.48v (http://imagej.nih.gov/ij). Statistical analysis of the effect of PCB 126 on cleavage rate and blastocyst rate, stage and grade was done by logistic regression (logistic procedure of SAS, Milltown, USA). Continuous variables were analysed in the GLM procedure. Replicate was considered as an influencing factor and was included in all models. The mean cleavage rate for the control group was 76.3% ±0.12 (mean ±SD) and in the PCB 126 treated group 70.0% ±0.09. Blastocyst rate (calculated from number of oocytes to maturation) on day 7 was higher in the control group (19.5% ±0.1) than the PCB 126 group (10.4% ±0.04). On day 8 the corresponding figures were 28.5% ±0.06 (control) and 21.4% ±0.04 (PCB 126 group). The difference in blastocyst rate between the control and the PCB 126 group was significant (p=0.04) on day 7, but not on day 8. There was no effect of PCB 126 on blastocyst stages, grades or number of nuclei. The mean pixel intensity of the LipidTOX stain was lower in the control group (334 ±139) compared to the PCB 126 group (454 ±212) but this was not statistically significant (p=0.18). In conclusion, addition of PCB 126 during maturation seemed to affect early embryo development in this small study, and could possibly be related to lipid metabolism. Bovine IVP should be further explored as a model for toxicity on oocytes.

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A272 Cloning, Transgenesis and Stem Cells

The morphology-based selection of mouse spermatogonial stem cells (mSSC) provides a cell population with pluripotency characteristics similar to mouse embryonic stem cells (mESC)

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Keywords: mouse embryonic stem cell, mouse spermatogonial stem cell, pluripotency.

The knowledge that mouse spermatogonial stem cells (mSSCs) present pluripotency characteristics similar to mouse embryonic stem cells (mESCs) opens a new path in cell therapy and regenerative medicine research. The aim of this work has been to verify if the morphology-based selection of mSSC provides a cell population with pluripotency characteristics similar to the mESC. The mSSCs were isolated from 5-10 day-old C57Bl/6 mice. The testicles were removed and the seminiferous tubules were treated with collagenase/DNase followed by trypsin. Following this, the cells were passed through a 70 µm nylon mesh and kept in culture in a 0.1% gelatin-coated dish. The cells were submitted to a sequence of incubation. The supernatant of the final incubation was collected and plated on a feeder layer composed of mouse embryonic fibroblasts inactivated with mytomicin C. The culture conditions for the mSSCs were 32°C in a 5% CO2 atmosphere and 90% humidity. The culture medium used was alpha modified MEM, supplemented with fetal calf serum (FCS), penicillin and streptomycin, non-essential amino acids, b-mercaptoethanol, N21, GDNF, EGF and bFGF. A lineage of mESCs (C57Bl/6), previously isolated in the laboratory, was used as a control group. The mESCs were cultured in DMEM high glucose, with FCS, penicillin and streptomycin, LIF, sodium piruvate, L-glutamine, b-mercaptoethanol and non-essential amino acids. The culture conditions for the mESCs were 37°C in a 5% CO2 atmosphere and 90% humidity. To analyze the morphology of the colonies, phase contrast microscopy was used and a DAPI/Phalloidin stain was performed. The pluripotency markers Oct-4 and Sox-2 were analyzed by immunofluorescence and the SSEA-1 expression was analyzed by flow cytometry. The morphology analysis by phase contrast and DAPI/Phalloidin staining showed that the mSSCs were organized in colonies like the mESCs. That can be considered as the first sign of mSSC pluripotency because it is the main morphological mESC characteristic. The immunostaining for Oct-4 and Sox-2 was positive in both groups, showing that two of the most important pluripotency transcription factors are present in the mSSCs. The SSEA-1 expression was only observed in the mESCs. However, in the literature it is related that the SSEA-1 expression in mESCs can vary and that it will probably not be the best pluripotency reference for comparison with the mSSC. These preliminary results showed that the isolation of mSSCs based on morphology leads to cells with pluripotent characteristics when compared with the mESCs. However, a more complete evaluation of pluripotency characteristics of these cells is needed, such as the production of embryoid bodies and the chimeras. Because mSSCs have no ethical problems compared to mESCs, they could be an interesting tool for use in cell therapy and regenerative medicine studies.

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A273 Cloning, Transgenesis and Stem Cells

Immunofluorescence and flow cytometry on characterization of equine induced pluripotent stem cells

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Keywords: flow cytometry, immunofluorescence, induced pluripotent stem cells.

The induced pluripotent stem cells (iPS) are adult cells genetically reprogrammed to a state similar to embryonic stem cells. Lentiviral vectors are used as a safe and effective way of producing iPS. The umbilical cord (UC) is a reserve of multipotent mesenchymal stem cell and therefore may represent an efficient source of cells to be reprogrammed. To characterize reprogrammed cells specific positive markers for pluripotent clones are used such as Oct-4, Nanog, Sox-2, TRA-1-60, TRA-1-81, SSEA-1, and SSEA3 SSEA-4. In this context, the aim of this study was to characterize, by immunofluorescence and flow cytometry methods, cells from equine cord matrix after reprogramming with lentiviral vector. For this, five samples of horses UC were collected at birth. Umbilical matrices were subjected to enzymatic digestion in a solution of 0.004% collagenase diluted in PBS, and cells obtained by filtration were plated into plastic culture flasks with 5 mL DMEM supplemented with 20% fetal bovine serum, antibiotics and antimycotics, followed by incubation at 37°C in a 100% wet, 5% CO2. When cells reached 40% confluence, a concentration of 10^5 cells were transfected with lentiviral human-vector EF1α 50mL STEMCCA (OKSM) (Millipore, SCR544), which contains the transcription factor OCT-4, SOX2, C-MYC and KLF-4 to generate iPS cells, according to the manufacturer's protocol, plus 8 ng/mL polybrene (hexadimethrine bromide, Sigma). The culture medium was renewed 12 hours after incubation. Five days after transduction, the cells were transferred into a subculture of mouse fibroblasts and cultured for 14 days in a specific medium for iPS. The first colonies were visualized after two weeks of the infection. When the clones were well established two mechanical passages and two enzymatic were made. After the re-establishment and proliferation of colonies, they were subjected to immunostaining protocols and analyzed by flow cytometry. Immunocytochemistry was performed in 6-well plates containing arround 20 to 30 colonies per well. For flow cytometry 200.000 cells were analyzed for each of the antibodies. In both techniques the following antibodies were used: Oct-4, Nanog, Sox -2, TRA- 1-60 and TRA- 1-81. We noticed that, in the immunofluorescence, horse umbilical cord cell's colonies scored positive for Oct-4, Sox-2 and TRA-1-81, as in flow cytometry, colonies were positive for Oct-4, Nanog, TRA-1-60 and TRA-1 -81. Although the markers used were specific to pluripotent cells, we did not observe a correspondence between the two techniques. Despite this, we can conclude that the equine umbilical cord cells were successfully reprogrammed and presented characteristics of pluripotent cells.

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A274 Cloning, Transgenesis and Stem Cells

Adult stem cells and animal reproduction: potential use of adipose MSCs as alternative to the traditional granullosa co-culture system in bovine IVEP

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Keywords: adipose mesenchymal stem cells, bovine IVEP, co-culture.

In bovine IVEP (b-IVEP), the use of co-culture system with granulosa cell monolayer is still very traditional. Somatic cells in co-culture would protect embryos from toxic metabolites and oxidative stress (Orsi, et al., Theriogenology (67), 441–458, 2007). It has been showed that MSCs are multipotent, secrete growth factors and cytokines, and can be isolated from many tissues of many species (Dimarino. Front. Immunol.(4) 201, 2013), including bovines. In this work we compared bovine adipose derived mesenchymal stem cells (b-AMSCs) and granulosa cells in co-culture system of b-IVEP. B-AMSCs were isolated from an adult female (collagenase type I - 1μg/mL) and cultured in IMDM with 10% FBS. At P3, stemness was evaluated by immunophenotyping (CD73, CD90 and CD105) and three-lineage *in vitro* differentiation (bone, adipose and cartilage). IVEP followed standard protocol. Oocytes were matured for 20 hours (TCM-199 with 10% FBS, FSH and LH) and fertilized for 24h with frozen semen from only one bull. Embryos were cultured in 100μL droplets of SOF medium with 5% FBS and 6 mg/mL BSA for 7 days in 5% CO2 at 38.5 °C. In experiment 1, two concentrations of b-ASCs were tested for co-culture: 10^3 b-AMSCs and 10^4 b-AMSCs. In experiment 2, embryos were cultured with 10^4 b-AMSCs, with IVM derived granulosa cell monolayer or without co-culture (Control). Cleavage and Blastocysts numbers were recorded on day 2 and 7 respectively. Blastocysts were analyzed for total cell number, and gene expression (POU5F1, G6PDHand HSP70). Results were analyzed by "t" test or ANOVA at 5% significance level. Stemness of b-AMSCs was confirmed at P-3 (CD73+, CD90+ and CD105+) as well as stem cells were successfully *in vitro* differentiated into adipose, bone and cartilage tissues. In experiment 1, the use of 10^4 b-AMSCs in co-culture improved ($p<0.05$; t test) blastocyst rate (54.16% ± 8.18) in comparison to the use of 10^3 b-AMSCs (33.11% ± 6.06). In experiment 2, blastocyst rate of co-culture with 10^4 b-AMSCs (45.97% ± 12.5; n= 231) was superior ($p<0.05$) to co-culture with granulosa cells (33,67% ± 14,7; n= 231) and Control (34.70% ± 9.8; n= 232). Moreover, co-culture with 10^4 b-AMSCs increased ($p<0.05$) total cell number of blastocysts (177.4 ±35.3; n=28) compared to co-culture with granulosa (136.2 ± 26; n=22) and Control (115 ± 21; n= 23). Finally blastocysts co-cultured with 10^4 b-AMSCs showed an increased expression ($p<0.05$) of POU5f1 (pluripotency) and G6PDH (glucose metabolism) in comparison to embryos co-cultured with granulosa cells. In conclusion, b-AMSCs (10^4 cells) can be successfully used for co-culture in b-IVEP. Compared to the traditional co-culture system with granulosa cells, co-culture with b-AMSCs improved quantity and quality of obtained embryos. Further studies must be done to evaluate the pregnancy rates of the embryos and to identify factors responsible for the effect of AMSCs on embryo development.

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A275 Cloning, Transgenesis and Stem Cells

Development of pig embryos produced by electrical or chemical parthenogenetic activation

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Keywords: chemical activation, electrical activation, parthenogenesis.

Parthenogenetic embryos produced after artificial activation of oocytes have high importance for the study of embryonic processes, including morphological and biochemical changes essential for development. The objective of this study was to evaluate the effect of parthenogenetic activation method (electrical or chemical) in pig oocytes on embryonic development rates. Cumulus-oocyte complexes (COCs) were recovered from slaughterhouse ovaries and matured in TCM 199 for 48 hours at 38.5°C and 5% CO2. After IVM, the COCs were denuded by repeated pipetting in TCM containing hyaluronidase and vortexed for 3 minutes. Chemical activation was performed with ionomycin (5 mM) for 5 minutes (Group IONO) and the two electric experimental groups, using 20V or 40V (both for 80 μsec), all structures were incubated in 6-DMAP (2mM) for 3 hours, followed by IVC in SOF for eight days. The cleavage and embryonic rates were assessed after three and eight days in culture, respectively. Data analysis was performed Fisher's exact test with $P < 0.05$. Out of 125 slaughterhouse ovaries, 567 COCs were obtained after puncture for IVM. Of these, 392 were matured resulting in maturation rate of 69.1%. These structures were used for parthenogenetic activation, activation being divided into three groups (IONO, 20V and 40V). In IONO, 20 V and 40V groups 153, 115 and 124 oocytes were activated, respectively. The activation rates in IONO, 20V and 40V groups were 74.5% (114/153), 80.9% (93/115) and 78.2% (97/124), respectively without any statistical difference ($P > 0.05$). The embryonic development rates in IONO, 20V and 40V groups were 21.1% (24/114), 17.2% (16/93) and 15.5% (15/97), respectively; showing no statistical differences between groups ($P > 0.05$). In conclusion, pig parthenogenetic embryos can be obtained efficiently by methods tested.

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A276 Cloning, Transgenesis and Stem Cells

Effect of trichostatin a in cloned cattle embryo production by nuclear transfer with mesenchymal stem cells

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Keywords: cloning, epigenetic, histone deacetylase.

Acetylation of histones is a major mechanism of genome epigenetic reprogramming of the gametes in order to establish a totipotent state to the normal development (Ikeda et al., Zygote 17, 209-215, 2009). The deacetylation is catalysed by histone deacetylases (HDAC) which remove acetyl groups and causes chromatin compaction and DNA segment silencing at this location (Johnstone, Nature Reviews Drug Discovery 1, 287-299, 2002). The trichostatin A (TSA) is an HDAC inhibitor that increases the amount of acetylated histones and the demethylation of DNA (Lee et al., Journal of Reproduction and Development 57, 34-42, 2011). In this sense, the drug has been used in an attempt to increase the production efficiency of embryos by nuclear transfer (NT). The objective of this study was to test the effect of TSA in exposure times of 20 and 25 hours in the culture of bovine embryos cloned by NT with mesenchymal stem cells (MSCs) derived from adipose tissue. A biopsy of skin and adipose tissue was collected from the perineal region of a female bovine Gir, at two months of age. The cells were isolated by the explant and cultured in Dulbeccos Modified Eagle Medium added with 10% fetal bovine serum. Cumulus-oocyte complexes recovered from slaughterhouse ovaries were matured for 18 h at 38.5°C and 5% CO_2. The NT was then performed with the adipose tissue and the MSCs reconstructed embryos were subjected to culture with 50 nM TSA for 20 and 25 h, for 4 h in activation medium containing 6-DMAP and further for 16 or 21 h in medium cultivation. Subsequently, the embryos continued in culture in synthetic oviductal fluid (SOF) medium without TSA and the parthenogenetic control was performed in every manipulation. Five NT procedures were performed for each treatment (20 h, 25 h and without TSA). Fusion rates, cleavage and blastocyst production were compared by Tukey test ($p < 0.05$). The cleavage rate of parthenogenetic embryos (93.45 ± 7.97) was higher than the cleavage rate of embryos without treatment (82.45 ± 5.59) but was statistically similar to embryos treated with 20 and 25 h (87.25 ± 8.41 and 85.54 ± 3.88, respectively). Still, there was no difference in cleavage rate between treated and untreated embryos. The blastocyst production rate on the seventh day of culture was superior to the parthenogenetic control (59.24 ± 11.75) compared to treatments for 20 h (36.22 ± 16.80), 25 h (33.66 ± 12.84) and without the use of TSA (32.70 ± 9.11), which did not differ. It can be concluded that the use of TSA had no significant effect in improving the rates of cleavage and development of bovine embryos by nuclear transfer with MSCs from adipose tissue. However, additional studies to evaluate the quality and embryos of the methylation pattern should be conducted to better understand the effects of TSA in embryos cloned with this cell type.

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A277 Cloning, Transgenesis and Stem Cells

Cell cycle effect and inhibition of deacetylation on development of swine embryos produced by nuclear transfer

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Keywords: cell cycle, cloning, scriptaid.

There is evidence from a number of studies in different species that treatment of somatic cell nuclear transfer (SCNT) embryos with inhibitors of histone deacetylase enzymes (HDACi) facilitates cell reprogramming and improves development. The aim of this study was to evaluate if the positive effect of HDACi treatment on SCNT embryos is affected by the cell cycle stage of nuclear donor cells and host oocytes at the time of embryo reconstruction. SCNT embryos were produced with MII or TII cytoplasts and G0-1 or G2/M stage nuclear donor cells. To obtain cells at G0-1 stage, fibroblasts were maintained in culture for at least 48h after reaching confluence and fixed for cell cycle analysis. The proportion of cells in G0-1 (88.2 ± 4.3%), S (2.7 ± 1.9%) and G2/M (9.1 ± 2.8%) confirmed that most of the cells were at the expected phase of the cell cycle. To obtain G2/M cells, confluent cells were trypsinized and plated in a non-confluent density, which allows for a synchronized wave of cells resuming their cell cycle and progressing to S and then G2-phases. The cells were then trypsinized and fixed for cell cycle analysis at 0, 16, 20, 24, 28, 32 and 36 h after plating. We confirmed that more than 30% of cells have reached the G2/M phase at 24 to 28 h post-passage from confluent cultures. Thus, we selected for nuclear transfer only the larger cells (mean diameter = 25.9μm) collected between 24 and 28h after plating from confluent cultures. Embryos reconstructed with the different cell cycle combinations were treated or not with the HDACi Scriptaid (500 nM) for 15 h, and then cultured in vitro for 7 days. A total of 568 embryos were reconstructed using G0-1 cells and MII or TII cytoplasts. Development to the blastocyst stage was significantly higher in MII (19.8%) than TII (4.6%) cytoplasts. Interestingly, Scriptaid treatment enhanced embryo development in MII cytoplasts (MII 19.8% vs. MII+S 32.6%; p=0.09), but not in embryos reconstructed with TII cytoplasts (TII 4.6% vs TII+S 6.2%; p=0.73). A total of 448 embryos were reconstructed using G2/M donor cells. Development to the blastocyst stage was significantly lower in MII (8.7%) compared to TII (22.2%) cytoplasts. Scriptaid treatment enhanced development in embryos reconstructed with MII (MII 8.7% vs. MII+S 16.6%) but not TII (TII 22.2% vs. TII+S 22.4%) cytoplasts. In summary, embryos reconstructed with MII-G0-1 and TII-G2/M developed to the blastocyst stage in higher frequency compared to the other groups, which confirms the importance of cell cycle interactions on cell reprogramming and SCNT embryo development. Treatment of reconstructed embryos with HDACi improved development of embryos produced with MII but not TII. Our findings indicate that: i) cell cycle interactions between the host cytoplast and the nuclear donor cell affect nuclear reprogramming; and ii) the response of SCNT embryos to HDACi treatment depends on factors present in MII-stage cytoplasts.

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A278 Cloning, Transgenesis and Stem Cells

Potential of completed conditioned medium (CM), supernatant (SN), microvesicles (MV) and lysed microvesicles (L-MV) derived from mesenchymal stromal cells (MSC) of equine amnion in suppressing allogeneic lymphocyte proliferation: preliminary results

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Keywords: cell, immunity, mesenchymal.

MSCs have been shown to secrete molecules with interesting paracrine effects and immunomodulatory properties that can be found in the medium where they are cultured. In addition to soluble factors, recent studies have suggested that some of the regenerative effects of MSCs are mediated by MVs. MVs can be classified as shedding vesicles released from plasma membrane or exosomes (endosomal membrane origin), and represent an important way of intercellular communication. The aim of this study was to evaluate the potential of the complete CM (soluble factors + MV), SN (soluble factors), MV and L-MV (internal content) derived from equine amnion MSCs (EqAMSC) in suppressing allogeneic peripheral blood mononuclear cells (PBMC) proliferation. Conditioned medium (CM) was obtained from the culture of $1x10^6$ equine amnion cells/ml after overnight serum privation culture. Microvesicles (MV) were obtained by ultracentrifugation of the CM. After centrifugation of the microvescicles, the supernatant (SN) was also recovered. Lysed Microvesicles (L-MV) samples were obtained after MVs' sonication. Three samples of each group were evaluated in triplicate. The different preparations mentioned above were added at different volumes and concentrations: CM and SN were plated 170µL, 120µL and 80µL, and MV was plated at $250x10^6$, $50x10^6$, $10x10^6$ and $2x10^6$ MV/well added to a culture of equine PBMC cells ($2x10^5$ in a final volume of 200µL of DMEM completed medium) in the presence of the mitogen phytohemagglutinin (PHA) at a final concentration of 2 µg/mL. Lymphocyte proliferation was assessed after 3 days of culture by adding 0.67 µCi/well of [3H]-thymidine for 16–18 hours and thymidine incorporation was measured using a microplate scintillation and luminescence counter. Next, to evaluate the effect of the internal content of MV on PBMC proliferation, L-MV samples were plated in the correspondent volume of three different concentrations of the entire MV resuspended in DMEM plated at $100x10^6$, $50x10^6$ and $5x10^6$ MV/well. Then, the PBMC proliferation assay was performed as already described. Paired T-test, baseline corrected unpaired T-test and ANOVA plus Tukey's test were used with GraphPad Prism software version 6 (Significance $P \leq 0.05$). The preliminary results obtained indicate that CM and SN were both able to significantly suppress more than 50% the PBMC proliferation when 170 µl and 120 µl were used; whereas none of the MV concentrations seemed to have any effect on lymphocyte proliferation, even after lysing. Based on these results, we conclude that MV and their internal content did not impact on lymphocyte proliferation, as shown by the CM and SN. However the immunomodulatory effects of MV cannot be ruled out since more studies are required regarding MV and L-MV influence on the different lymphocytic subpopulations.

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A279 Cloning, Transgenesis and Stem Cells

Clones embryo production in deer *(Mazama gouazoubira)* using goat oocytes: preliminary results

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UECE.

Keywords: deer, goats, iNTSC.

The brocket deer (*Mazama gouazoubira*) is a species of deer present in northeastern Brazil. Due to the decrease of its territory and poaching, among others, this species may be threatened in the coming years. Furthermore, it can be used as a model for the maintenance of species already endangered by the use of interspecific reproductive cloning. The aim of this study was to produce interspecific Nuclear Transfer Somatic cell (iNTSC) clones embryos (deer and goats) and evaluate their in vitro embryo development. Intraspecific cloned embryo (goat and goat) were used as control. Fourteen goats were used as oocyte donors after hormone treatment consisting progestin / luteolytic and FSH shortly after the end of treatment. After ovarian puncture, the oocytes were evaluated for IVM and placed in TCM 199 supplemented with 10% FBS for 22-24 hours in humidified atmosphere at 38.5°C and 5% CO2. After IVM, oocytes were denuded and stained with Hoechst 33342 for enucleation using an inverted microscope (Nikon TE2000, Tokyo, Japan) equipped with micromanipulators (Narishige, Tokyo, Japan), under UV light. Previously, goats and deer fibroblasts were grown to 95% confluence, and used as karyoplastos to NTSC and iNTSC respectively, in the passage 3-7. The embryos were reconstructed by electrofusion (Multiporator, Eppendorf, Hamburg, Germany) using ionomycin-DMAP activation procedure. Those in which the reconstruction was confirmed were cultured in vitro for eight days in mSOF supplemented with 5% FCS in a humidified atmosphere at 38.5°C with 5% CO2, 5% O2 and 90% N2 (Eve, WTA, Craven , Brazil). The embryonic development rates were evaluated by Fisher's exact test with $P < 0.05$. From 28 ovaries 124 oocytes were obtained, which resulted in an average of 4.4 oocytes per ovary. Out of oocytes harvested 56 (45.0%) were considered grade I and placed to IMV. The maturation rate was 75.0% (42/56). Out of the matured oocytes, 24 were used for iNTSC and 13 for NTSC. Cleavage rate of 38.4% and 25.0% for iNTSC and NTSC embryos was obtained, respectively, ($P > 0.05$). The blastocysts rate in iNTSC group was 50.0% (3/6) and in NTSC was 40.0% (2/5), with no significant difference ($P > 0.05$). These preliminary results show that goat oocytes have the potential to be used as cytoplasts for deer in future conservation programs.

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A280 Cloning, Transgenesis and Stem Cells

A reliable protocol to synchronize fibroblast cells at the G2/M phase

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Keywords: Cdk1, cell cycle, ro-3306.

Cell cycle coordination is important to investigate nucleus-cytoplasmic interactions after cell fusion and nuclear transfer in basic research. However, there are no reliable protocols that result in higher rates of cells synchronized at the G2/M phase during in vitro culture. The aim of this study was to evaluate the efficacy of a CDK1 inhibitor (RO3306) to synchronize fibroblasts cells at G2/M phase. Porcine fibroblast cells were maintained in culture until reaching confluence and then used to prepare non-confluent cultures by plating 50,000 cells per well of a 6-well plate in 2 ml of culture media. Cells were then trypsinized and fixed for cell cycle analysis at 0, 16, 20, 24, 28, 32 and 36 h after plating. A non-confluent density of cells starting from a confluent culture allows a synchronized wave of cells resuming their cell cycle and progressing to S- and then G2-phases. Indeed, we observed that more than 30% of the cells reached the G2/M phase at 24 to 28 h post-passage from confluent cultures. Based on these results, we tested the effect of two doses (5µM and 9µM) of RO3306 starting at 16 or 20h post-passage from confluent cultures. Cells were fixed to determine their cell cycle stage at 24, 28, 32, 36 and 40h after plating. For cell cycle analysis, cells were trypsinized (0.25% trypsin-EDTA) and re-suspended in ice-cold fixation solution (70% ethanol and 30% PBS) for 15 minutes. Fixed cells were then pelleted by centrifugation and re-suspended in PBS. Before flow cytometry analysis, cells were re-suspended in 1ml PBS containing 50µg propidium iodide and 100µg RNAse at 37˚C for 40 minutes. DNA content of 10,000 cells was determined by fluorescence-activated cell sorting (FACS) using a FACSVerse system (BD Biosciences, San Jose, CA). The percentage of cells at G0-1, S or G2/M was calculated using the FACSsuite Software (BD Biosciences). When treatment started at 16h post-passage, the highest proportion of cells synchronized at the G2/M phase (41.9%) was observed in the group exposed to 5µM RO3306 until 32h, which was significantly higher than control cells fixed at the same time (22.2%). When treatment started at 20h post-passage, higher percentages of G2/M cells were obtained in the groups treated with 9µM RO3306 and fixed at 36h (61.7%) and 40h (56.1%) post-passage. The diameter of cells that were at the different cell cycle stages was determined in cells treated with 9µM RO3306 and fixed at 36 or 40h post-passage. The mean diameter of cells in G1-0 and G2/M phase was 13.9µm and 25.9µm, respectively. Our results provide a reliable methodology based on CDK1 inhibition and cell diameter for selection of G2/M cells.

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A281 Cloning, Transgenesis and Stem Cells

Somatic cell nuclear transfer in the equine with donor cells in G2 and oocytes in telophase II

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In Vitro Clonagem.

Keywords: clone, equine, somatic cell.

Equine production by somatic cell nuclear transfer (SCNT) has only been reported by a few labs due to the lack of available equine oocytes and the high cost of maintaining and working with equine. The protocol commercially used for cloning equine by our competitors was adapted from the procedure used to clone Dolly the sheep. In 2005 In Vitro Clonagem (IVC) developed a novel procedure for cloning bovine which we later adapted for use in equine. The Dolly protocol uses donor cells in G1 or G0 of the cell cycle and enucleated metaphase II oocytes whereas the In Vitro Clonagem protocol uses donor cells in G2 of the cell cycle paired with enucleated oocytes in Telophase II of meiosis. Here we compare the procedure used to clone Dolly with the IVC novel cloning technique, both adapted for use in the equine. Our results demonstrate a similar rate of cleavage and a superior rate of blastocyst production and initial pregnancy in comparison with the Dolly cloning method. Overall, we observed: 1. Cleavage rates of embryos were similar between both protocols. 2. The observed increase in blastocyst rate of the IVC over the Dolly method of cloning was statically significant. 3. The observed increase in pregnancy rate of the IVC cloning method over the Dolly cloning method was statically significant. 4. We lost the pregnancy established using the Dolly method of cloning whereas we have four foals born from the IVC cloning method. We have successfully adapted our bovine cloning method using oocytes in Telophase II and donor cells in G2 phase of the cell cycle for use in the equine. The adapted protocol outperforms the method used to clone Dolly in terms of embryo development to the blastocyst stage and establishing pregnancies. Although we are still waiting for live birth results, we have already delivered four cloned equine and are waiting for more to be born in the year ahead.

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A282E Cloning, Transgenesis and Stem Cells

Metabolomic analysis revealed differences between bovine cloned embryos with contrasting development abilities

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Keywords: bovine embryo, cloning efficiency, genotype.

Cloning by somatic cell nuclear transfer has been established in various species but its efficiency remains low. Efforts have been made to improve cloning efficiency mainly focusing on reprogramming, using donor cells sourced from different somatic tissues and using epigenetic modifiers. Differences in reprogramming efficiency linked to the genotype of donor nuclei have been observed in mouse and bovine, but remained largely unexplained, especially in the bovine. This study is part of a set of analysis to understand the early differences between two types of bovine cloned blastocysts with different term development abilities (2.5 versus 12.5% birth rates) (Bui, Reproduction, 138(2), 289-99, 2009). Blastocysts were obtained by nuclear transfer of fibroblasts derived from ear skin of two different Holstein heifers (OV 0029 and OV5538) as described (Khan, PlosOne, 7(3), e34110, 2012). After activation, embryos were cultured in groups in SOF medium with 1% oestrus cow serum (39°C, 5%CO2, 5%O2). On Day 6 embryos were cultured individually for 24 hours in 12µl droplets of SOF (Minitube) plus Bovine Serum Albumin. On Day 7, 10µl spent culture medium (CM) and blank samples were collected and frozen until Fourier Transform Infrared Spectroscopy (FTIR) analysis. The metabolic fingerprint of spent CM of the two types of cloned blastocysts were compared. Briefly, samples (n=36 and 65, respectively for OV0029 and OV5538) were analyzed using a Golden-Gate ATR device mounted on a Varian 620-IR FTIR spectrophotometer. After correction for experimental series and subtraction of values obtained for blank samples from experimental values, the FTIR spectra were analyzed by redundancy analysis (RDA), a method which combines multiple regression with Principal Component Analysis. The model included embryo stage at day7, embryo grade at day7 (according to IETS grading; only grade 1 and 2 embryos) and embryo genotype. Significance of the effect "embryo genotype" was addressed with permutations tests. Analysis on the whole spectra did not show significant effects. However, focusing on sub-regions of the spectra pointed to a significant "embryo genotype" effect (p=0.035) for wavelengths between 2850 and 3030cm^{-1}. The above identified region of the spectra covers the CH_3 and CH_2 asymmetric stretching from lipids (Socrates, Infrared and Raman Characteristic Group Frequencies, Tables and Charts, Ed. Wiley, 2001 ch 23). Therefore, our results suggest differences in lipid metabolism between these two types of clones. Further analysis is in progress to confirm this hypothesis.

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A283E Cloning, Transgenesis and Stem Cells

Embryo collection in clone cattle offspring

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Keywords: cattle, cloning, embryo collection, offspring, superovulation treatment.

Our laboratory has been working on bovine clones for many years. These clones were studied and several cloned females were bred to obtain clone offsprings. All pregnancies were normal and calves developed as healthy individuals. The females were used for embryo collection after superovulation. The objective of this study was to compare the embryo recovery results between clone offspring and control animals. Altogether, 28 cows were used for this study (18 clone offspring and 10 controls). All the animals were born and raised in the same experimental farm, in the same time period and in the same rearing conditions. 90 flushes were performed to collect D9 to D21 embryos for research protocols. For early embryos on D9, a classical 3 way collection equipment (IMV, France) was used. To collect the late embryos D12-D21, the same equipment was modified so that larger embryos could be collected through the remaining larger hole (2 way collection) (Richard et al. 2015, Theriogenology 83, 1101-9). All females were submitted to ovum pick-up to remove the dominant follicle and were subsequently superovulated with FSH (Stimufol®, Reprobiol, Belgium). Luteolysis was induced 48 hours prior to AI. Two AI were performed with frozen semen, 48 and 56 hours after PGF2α injection (Estrumate®, MSD Santé Animale, France). Before embryo collection, cows were treated with an epidural injection of 3-4 ml (Xylovet®, CEVA Santé Animale SA, France).The presence of Copora Lutea (CL) was checked and they were counted by rectal palpation. For all collections, the cervix was prepared with the initial introduction of a dilator. Then the catheter was introduced in one horn and the cuff was inflated as low as possible. For the collection of late stage embryos, 30 ml (Euroflush, IMV, France) was injected slowly twice to suspend the embryos prior to flushing the horn with 500 ml, and the same operation was performed on the second horn. Data were analyzed by unpaired t-test using Prim® software. There was no significant difference in the number of embryos collected per flush in clone offspring and controls (349 embryos collected, 5.05 ± 4.8 per flush vs 90 embryos collected, 4.28 ± 3.92 per flush, respectively). The number of CL was also not significantly different between groups (11.49 ± 7.32 and 8.43 ± 4.26 per flush, respectively). For late collections in all animals, the FSH dose (Stimufol®) was reduced to limit the number of embryos and preserve development (Richard et al. 2015). Retrospectively there was no significant difference for the necessary dose for superovulation (0.57 ± 0.08 for clone offspring and 0.54 ± 0.07 for controls). These data indicate that offspring of clones raised since birth in the same conditions as control heifers have the same ability to give embryos after superovulation treatment indicating equivalence of reproductive function.

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A284 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Bioinformatic approach to establish predictors of oocyte development competence in cumulus cells

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Keywords: biomarker, cumulus, oocyte.

One of the greatest difficulties in assisted reproduction techniques is the selection of high-quality oocytes for further fertilization and implantation. Consequently, the search for oocyte quality bioindicators is constant. Cumulus Oophorus is formed by a group of somatic cells surrounding the oocyte. These cells have been widely studied because of their intimate relationship with the oocyte during the entire folliculogenesis, oocyte maturation and ovulation processes. Cumulus cells gene expression and biochemistry are influenced by oocyte condition, as well as both follicular and ovarian environment. Still, these cells are discarded after in vitro fertilization techniques. This makes them an easy-to-access material, which can be obtained non-invasively and free of ethical biases. Our aim in this study was to identify, at cumulus cells level, oocyte competence related genes. To achieve this objective, we searched public repositories (Gene Expression Omnibus) for gene expression data from microarray obtained from cumulus cells experiments and which also provided clinical and pathological descriptions of the samples, including end-points of the germinative cell (cleavage, blastocyst formation, implantation). In these databases, we found patients samples comparing cumulus from follicles that originated good and bad quality oocytes (GSE55654, GSE54135, GSE37277, GSE22869, GSE18559, GSE9526), and also patients' samples with different pathological conditions, such as obese and polycystic ovary patients (GSE10946). To evaluate differences between experimental groups in these databases and obtain information about oocyte competence, we used three computational approaches: 1) differential expression analysis at R statistic environment using LIMMA package; 2) pathway enrichment utilizing GSEA (geneset enrichment analysis) method; and 3) Biological processes enrichment utilizing the online tool DAVID. Moreover, to reinforce and corroborate this approach and its results, we also used animal assisted reproduction studies (GSE65269, GSE36605, GSE31261). Our results, in addition to establish oocyte quality biomarkers, highlight the relevance of using cumulus cells to identify oocyte quality.

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A285 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Combination of hCG and deslorelin acetate on the induction of ovulation in mares: changes in follicular fluid protein profile

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Keywords: folicular fluid, ovulation-inducing, proteomic profile.

The composition of follicular fluid (FF) is essential for the proliferation and differentiation of granulosa cells in addition to processes related to rupture of the follicular wall, maturation and fertilization of the oocyte and luteinization. In the mare, there are few studies evaluating FF proteome (FAHIMINIYA, Prot Sci, 9:1, 2011; PETRUCCI, J Eq Vet Sci, 34:115, 2014). The ability to induce ovulation in a reliable way is important in equine reproductive management in different situations the main ovulation-inducing agents used are hCG and deslorelin. The aim of this study was to compare the protein profile of FF on induced ovulation of mares with hCG or with the combination of hCG and deslorelin acetate. Fourteen mares were used (3-12 years). Following the observation of follicles ≥ 35 mm and with endometrial edema, the mare was submitted to the induction protocols: Group H 1000 UI, IV, of hCG or Group HG 1000 UI hCG, IV, + 1,5 mg of Deslorelin acetate, IM. In the subsequent cycle mares were submitted to a protocol different from the previous cycle. Samples were collected by transvaginal aspiration 32 h after induction and submitted to the quantification of proteins by the Bradford method. Two-dimensional electrophoresis was performed in 12.5% polyacrylamide gel and stained with Coomassie G250, scanned and analyzed using PDQuest v.8.0.1. Spots with significant differences in relative abundance between group H and HG were cut out submitted to trypsin digestion and mass spectrometry. In this study the total protein concentration in the mare FF from Group HG were higher (73.07 ± 6.42 mg/ml) than those induced with hCG alone (63.97 ± 6.97 mg/ml). Comparative analysis showed a significant difference in the abundance of five spots between groups. Two Alpha-1-antiproteinase 2 (A1AT2), the Serotransferrin (TF) and Antithrombin III (ATIII) had lower relative expression in group HG and the Haptoglobin (HP) showed greater abundance in the same group. The lowest expression of A1AT2 at the final moment of follicular maturation prior to ovulation is likely related to the need for lower inhibitory action on the proteolytic activity allowing fine adjustment that controls the ECM degradation, inflammation and the coagulation cascade (BIANCH, J Prot, 90:61, 2013). ATIII is also serine-type endopeptidase inhibitor activity. The last protein less expressed in the group HG was TF. Increase in cellular iron levels stimulates the expression of some MMPs that degrade the ECM. There are reports of increased transferrin in granulosa cells and oocyte follicles in more advanced stages of maturation, which could explain the reduction in FF transferrin. Haptoglobulin showed increased abundance in the group HG and exerts anti-inflammatory action due to inhibition of oxidative damage. These proteins are probably related to the final events of oocyte/follicle maturation that trigger ovulation and subsequent luteinization.

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A286 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Evaluation of *in vitro* embryo production rate and re-expansion post thawing with two different culture media

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Bio Biotecnologia Animal.

Keywords: cryobiology, cryopreservation, cryotolerance, vitrification.

The in vitro embryo production is widespread in Brazilian dairy and beef cattle. However, the cryopreservation of IVF embryos remains a major step to be done for the IVF employment in an exponential scale. The aim of this work was to evaluate the effect of supplementing in vitro culture with BSA during the first 72 hours of bovine in vitro culture (IVC), on their embryonic development and cryotolerance. Slaughterhouse-derived oocytes were matured and fertilized in vitro according to standard procedures. Twenty-four hours after in vitro fertilization, zygotes were cultured in two different groups: Group Control, IVC media supplemented with 10% BFS from D.0 to D.7; Group BSA-BFS, from D.0 to D.3, IVC media + 0.6% BSA and, D.3 to D.7, IVC media + 10%SFB. Embryo development rates were evaluated on D.7, when the expanded blastocysts (Stage 7) were vitrified. Embryo cryotolerance was evaluated 48 hours after thawing, by the percentage of embryos resuming development to reach a more advanced stage, and hatching rate. BSA during the first 3 days decreased the blastocysts yields comparing with Control Group [23%a (146/637) vs 34%b (236/700), respectively; $p \leq 0.01$]. However, BSA improved embryo cryotolerance compared to the Control Group, due the higher development and hatching rates [88%a (63/72) vs 77%b (66/86), respectively; $p \leq 0.05$] after 48 hours post-warming culture. In conclusion, although the use of BSA during the first 72 hours of IVC decreases the embryo production, it improves the embryo cryotolerance.

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A287 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Evaluation of DNA amplification kits for genomic analysis of cell biopsies from *in vitro* produced bovine embryos

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Keywords: biopsy, embryo, genomic.

Cattle producers are continually looking for ways to increase the number of genetically superior animals, while at the same time reducing production costs. A genetic screen of in vitro produced embryos for traits of economic importance, prior to transfer, would greatly benefit genetic selection and reduce costs. This technique involves removal of a few cells from the growing embryo at the morula or blastocyst stage. The limitations of whole genome analysis (WGA) of embryo biopsies is to balance the number of cells that can be removed without compromising the embryo and obtaining sufficient amounts of DNA. With the development of molecular biological techniques such as DNA amplification, these limitations are no longer an issue. The objectives of this project were to evaluate: 1) the effects of biopsy and vitrification in a 2x2 factorial design on embryo re-expansion, and 2) the call rates of DNA from embryo biopsies that were amplified with commercially available DNA amplification kits. In experiment 1, at day 7 of in vitro production, bovine embryos either not manipulated (C) or subjected to embryo biopsy (B), in which around 10 cells from the trophoblast layer were removed. Both groups were either placed back into culture for 18 hrs (Fresh: C/F and Biopsied/Fresh: B/F) or cryopreserved by vitrification (C/V and B/V). Vitrified embryos were then thawed and placed into culture for 24 hrs. Re-expansion was evaluated in all groups. In experiment 2, DNA from biopsied embryos was amplified by GenomePlex Single Cell WGA (Sigma), REPLI-g Single Cell (Qiagen) or Illustra Single Cell GenomiPhi (Ge Healthcare) kits. Whole genome analysis was performed on the Geneseek Genomic Profiler LD (GGP-LD) for Indicus-35,000 SNP Platform (Neogen). One-way ANOVA test was utilized to determine significance ($P<0.05$) for re-expansion and call rates and differences in means was determined by Tukey`s. Re-expansion of B/F embryos (93.3+4.6%) was no different than C/F group (100%). Re-expansion of B/V embryos (75.0+6.7%) was lower than C/F, with C/V embryos having the lowest re-expansion rates (52.2+ 15.5%). There was a significant difference in call rates for DNA amplified using the REPLI-g (86.6+5.2%) and GenomiPhi (59.9+7.8%; $P<0.05$) kits. The GenomePlex kit (82.7+2.1%) was not different than the other kits. These results suggest that whole genome analysis of embryo biopsies is possible with DNA amplification without compromising embryo survival.

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A288 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

In vitro evaluation of hyperactivation induction with procaine and calcium ionophore in cryopreserved equine sperm

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Keywords: freezing, frozen semen, sperm capacitation.

The aim of the present work was to test 2 in vitro hyperactivation protocols of cryopreserved equine sperm. After semen thawing, the sperm were selected by swim-up and distributed in 3 aliquots according to the treatments T1) Control: capacitating Whitten's Medium (WMc), T2) Procaine: T1 + 5mM Procaine chloride (Sigma-Aldrich, St Louis, MO, USA) e T3) Calcium Ionophore: T1 + 5µM Calcium Ionophore A23187 (Sigma-Aldrich, St Louis, MO, USA). After 10 min at 37°C, the samples were diluted to10x106 sperm/mL and the sperm motility was verified by a computerized assisted sperm analysis (CASA). To the bovine zona-binding assay (ZBA), bovine oocytes derived from abattoir ovaries were stored in a hyperosmotic solution (Yanagimachi et al., Fertil Steril, v.31, n.5, p.562-574, 1979), and the equine sperm, in the 3 before mentioned treatments, were stained with 35µg/mL Hoescht 33342 dye (Sigma-Aldrich, St Louis, MO, USA) and resuspended with WMc to 2x106 sperm/mL. The oocytes were incubated at 38.5°C with 5% CO_2 for 1 hour and each 5 oocytes were poured into each treatment droplet under mineral oil (Coutinho da Silva et al., Reproduction, v. 143, p. 577–585, 2012). After 2 hours of co-culture, the number of sperm attached to the ZP was determined with epi-fluorescent microscopy. The experimental design was performed completely randomized. Means of zona pellucida (ZP) attached sperm were compared by ANOVA and Tukey Test, the VCL, VSL, VAP, LIN, STR sperm characteristics by ANOVA, Tukey test and Scott-Knott, and ALH and BCF by Friedman's Test. The complete data were analyzed using the SAS program. A probability of $P<0.05$ was considered significant. Lower number of ZP attached sperm was observed by the calcium ionophore induced hyperactivation protocol (1.9±2.1) compared to the procaine (5.9±3.7) and control (5.7±3.6, $P<0.05$). The procaine hyperactivated sperm showed the lowest VSL, VAP e LIN values, while ALH e BCF did not differ from the control and calcium ionophore hyperactivated sperm ($p>0.05$). It can be concluded that equine cryopreserved sperm were better hyperactivated with procaine than with calcium ionophore, and therefore, it is a more suitable sperm hyperactivation inductor to study equine IVF protocols with cryopreserved semen.

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A289 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Lipid characterization by Maldi-MS of *in vitro* produced bovine embryos with different developmental kinetics

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Keywords: bovine embryo, lipids, mass spectrometry.

Embryo studies have shown that morphological differences related to the moment of the first embryonic cell divisions and its kinetic (fast and slow), appear to be relevant to the embryo viability (Meseguer, M, Hum. Reprod, 26, 2658-71, 2011; Lundin, K, Hum. Reprod, 16, 2652-2657, 2001). Embryonic viability can also be severely compromised by lipid accumulation in IVP embryos and can even harm commercial applications such as cryopreservation (Abe, H, Mol. Reprod.Dev, 61, 57-66, 2002). Therefore, the aim of this study was to evaluate the relation between developmental kinetic and the lipid profile on bovine embryos. For this goal, IVP embryos were produced and, 18 hours post insemination (hpi), the presumptive zygotes were individually cultured for sequential medium (established in previous studies). First, they were cultured for 22 hours in KSOM (Millipore®) [supplemented with 10 % of FCS, 0.25µL/mL of gentamicin and 4.5µL/mL of nonessential amino acids] and classified as fast (F - 4 or more cells) or slow embryos (S - 2-3 cells). The medium was then replaced by SOF [containing 5% of FCS, 10µL/mL of nonessential amino acids and 20µL/mL essential amino acids] and the embryos were IVC until blastocyst stage (168hpi). The study also included a group of *in vivo* (VBL) embryos at the blastocyst stage. The lipid characterization, by MALDI-MS, was performed at 40hpi (FCL vs SCL), 96hpi (FMO vs SMO) and 168hpi (FBL vs SBL vs VBL). The lipid characterization data obtained was submitted to multivariate statistical analysis, by Partial Least Squares- Discriminant Analysis (PLS-DA), using the MetaboAnalyst 3.0 website. The ions highlighted were subjected to ANOVA with subsequent Tukey test (GraphPad Prism Software Inc 5), to show their relevance in the study groups. Fast and slow groups presented different relative abundances of membrane sphingomyelins (SM) and phosphatidylcholines (PC) by MALDI-MS at 40 hpi (of *m/z*: 725.5, 732.6, 758.6, 788.6, 808.6, with $p<0.05$) and 96hpi (of *m/z* 706.5, 725.5, 782.6, with $p<0.05$). Regarding the blastocyst stage, the slow group presented a higher relative abundance of the SM and PC species, which are similar to the VBL group (of *m/z* 703.5, 760.6, 784.6 and 808.6, with $p>0.05$). The slow group still showed some similarities with the fast group, which may be due to the exposure to the same *in vitro* environment (SM of *m/z* 703.5 and PC of m/z 734.6, for 96 hpi. PC of *m/z*: 786.6 and 808.6, for 168 hpi, $p>0.05$). In addition to their structural function, these lipid classes have an important role in cell signaling such as stress, pregnancy and cryopreservation. The ion data obtained for the PCs and SMs classes, allowed us to infer that there are more similarity between the SBL and VBL.

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A290 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Characteristics of H3K4me3 in bovine embryos produced *in vivo* with sexed semen and submitted to cryopreservation

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Keywords: epigenetics, histone methylation, sexing of semen.

Cryopreservation of embryos produced *in vivo* is considered efficient and the knowledge of its basic biology can be used as a parameter for better understanding of the mechanisms involved in the low efficiency of cryopreservation in embryos produced in vitro. The objective of this study was to characterize the modifications of tri-methylated lysine 4 on histone 3 (H3K4me3) in bovine embryos produced in vivo with sex-sorted semen and subjected to cryopreservation. Girolando cows (a common Brazilian dairy breed, crossbreed Gir x Holsteins; n = 5) were superovulated and inseminated with sex-sorted or conventional semen. After the embryo collection (n = 4), aliquots of the collected embryos were stored in Paraformaldehyde immediately after collection, or stored in the same medium after cryopreservation / thawing. All embryos (fresh / conventional semen, fresh / sexed semen; cryopreserved / conventional semen and cryopreserved / sexed semen) were evaluated by immunofluorescence in confocal microscopy to identify the H3K4me3. From 190 embryos collected, 100 were produced with conventional semen and 90 with sexed semen. The use of conventional semen provided 72% of viable embryos, the majority (52.77%) of them was in more advanced stages of development (expanded blastocysts; 52.77%). In contrast, embryos produced with sexed semen showed lower viability rate (36.7%) and earlier stages of development at time of collection (morula and blastocyst, 42.42%; $p < 0.05$). Embryos in morula stage, regardless of cryopreservation or the use of sexed semen, showed the most prevalent signal of the H3K4me3 compared to blastocysts (209.26 ± 10.95 vs. 160.35 ± 10.52 intensity channels; $p < 0.05$). These are the first results that describe the H3K4me3 profile of bovine embryos produced in vivo. As well fresh embryos as cryopreserved, and produced with conventional or sexed semen showed the same pattern of trimethylation of H3K4, but in morula the abundance was more pronounced.

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A291 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Lysine 4 of histone 3 trimethylation characteristics in bovine oocytes matured *in vitro* and cryopreserved - preliminary results

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Keywords: cryotop, epigenetic, H3K4.

It has been reported that immature oocytes are more sensitive for vitrification then mature. Factors such as maturity may change the pattern of DNA methylation and histone, compromising embryonic development. Accordingly, it is believed that the cryopreservation of oocytes can be influenced by epigenetic changes. Therefore, the aim of the study was to characterize the pattern of trimethylated H3K4 (H3K4me3) in mature and immature oocytes before and after vitrification. Oocytes were obtained by follicular puncture of bovine ovaries from slaughterhouse, later screened and classified according to their morphology and then divided into four groups: Group 1 = immature fresh (n = 15); Group 2 = immature cryopreserved (n = 15); Group 3 = mature fresh (n - 15); Group 4 = mature cryopreserved.The groups 3 and 4 were matured for 24 hours in incubator (5% CO_2, 38.5°C). Groups 2 and 4 were vitrified by cryotop method. All groups were kept in 3% PAF at 4°C to evaluate the H3K4me3, the oocytes were exposed to polyclonal primary antibody against H3K4me3 (CellSignaling®; 1:1000), and secondary antibody (anti-Rabbit Alexa-647; Invitrogen A21245, 1:200), and then subjected to staining of the nucleus (SYBR Green, Molecular Probes, Goettingen, Germany; 1:500). Finally, the oocytes were evaluated under confocal microscopy. The signal was analyzed by using the ImageJ program. Data were analyzed by ANOVA (factorial), and when necessary carried out Tukey test at 5% probability (R Program). The mature oocytes showed higher signal intensity (193.43 pixels) that immature (149.00 pixels) ($p<0.05$) different than expected, since the transcript in that period is known to be reduced and also supported by the fact that DNA methylation is high in mature oocytes. Fresh oocytes (the ones that have not undergone vitrification), on the other hand, showed higher signal intensity (178.00 pixels) than vitrified oocytes (164.43 pixels) ($p<0.05$). This fact was expected, as cryopreservation can cause injuries that culminate with the lowest transcriptional activity, which could interfere with the quality of oocytes and their ability to become a viable embryo. Hypothesis supported by the lower embryonic development rate of cryopreserved / thawed oocytes. The interaction of the variables was not observed ($p>0.05$). We can conclude, therefore, that maturation and cryopreservation of bovine oocytes affect the trimethylation of H3K4.

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A292 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Comparison of pregnancy rates after fresh, vitrified or cryopreserved *in vitro* produced embryos for direct transfer

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Keywords: cryopreservation, direct transfer, IVF embryos.

Although over 30% conception rates have recently been achieved from embryo transfer (ET) of vitrified in vitro produced (IVP) embryos, the complex process of recovery of these embryos after vitrification remains an obstacle to commercial use of this technique, with little applicability under field conditions. The aim of this study was to compare pregnancy rates obtained after ET of fresh IVP bovine embryos, vitrified or frozen for direct transfer in the commercial routine of a big dairy farm. Oocytes (n=3171) recovered by OPU from 120 Girolando females were selected and submitted to IVM for 24 hours at 38.5°C with 5% CO_2 in air and saturated humidity. The IVF was performed with sexed semen thawed from 5 Holstein bulls. After IVF, the presumptive zygotes were denuded and cultured for seven days under the same conditions for temperature and humidity as IVM and IVF, but with 5% CO_2 and 5% O_2. Grade I embryos in stages BL or BX were transferred to fresh, frozen or vitrified for direct transfer (DT). Embryos were transferred into previously synchronized recipients. The pregnancy rates was analyzed by Binomial Logistic Regression and the probability level of $p<0.05$ was considered significant. Conception rates obtained were 51.35% (133/259) for embryos transferred fresh, 34.62% (84/234) for vitrified and 42.11% (96/228) for direct transfer embryos. The rates obtained from IVP embryos vitrified and direct transfer were not different between these two groups, but they were lower than the pregnancy rates from the fresh group. These results indicate that cryopreservation of IVP embryos yields results similar to those obtained after transfer of IVP fresh embryos. These results highlight the positive aspects of the possibility of cryopreservation of IVP embryos with the convenience of direct transfer.

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A293 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Comparison between Bioxcel® and Tolera-D® semen extenders on semen industrialization rates

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Keywords: cryopreservation, flow citometry, semen.

Male fertility is always put to the test when undergoing a bull was submitted for semen collection and cryopreservation processes. There are several different factors that interfere on this; the quality of semen extender is one of them. The goal of this paper was compare de approval semen industrialization taxes (refused versus approval semen bath for commercialization) process with two different commercial semen extenders (Bioxcell® and Tolera-D®). Six semen donors were used (03 Nellore, 01 Angus and 02 Senepol bulls), collected by electroejaculation or artificial vagina. The ejaculates were evaluated by volume, mass motility, motility, vigor and morphology. After initial approval the ejaculates were split in 2 equal parts, one diluted with Tolera-D® (Inprenha, Jaboticabal, Brasil) and another with Bioxcell® (IMV, L'Aigle, França). After dilution, the semen bath was submitted by cooling, equilibration and freezing curve routinely used by the company. Semen was frozen in 0.25mL semen straw. Four straws randomly choose for each semen bath were chose for evaluation. After thaw (35oC in water), the semen batch were analyzed in contrast microscopy (100x and 1000x) by motility/vigor at thaw (MOT/V0h), motility/vigor after 3hs at 37ºC incubation (MOT/V3h), motility/vigor after 30' at 45ºC (MOT/V30) and % of viable sperm cells by flow cytometer-MUSE® (%VIACOUNT). The VIACOUNT test was design following the manufactory recommendations, with dilution factor (121X) and 10.000 events considered. The acceptable pattern to approval frozen semen batch was MOT/V0h >40/4; MOT/V3h >20/2; MOT/V30 >20/2; % VIACOUNT >40%; Concentration >20x106sperm/dose, total morphological defects <30% or major defects <20%. The approval rates of semen freezed using Tolera-D was 83% (100% in Nellore and Angus bulls and 50% of approval in Senepol breeder). Even the Bioxcell approval semen industrialization rates were 67% (83% in Nellore, 0% Angus and 50% in Senepol bulls). The results showed no statistical difference between extender's and approval semen industrialization taxes (Bioxcell® ou Tolera- D®), but we just note some particularities between some individuals and his semen behavior using different extenders. Both extenders have similarities between chemical composition and crioprotectors (glycerol and soybean lectins) and they demonstrated good and similar approval semen industrialization tax.

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A294 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Cryopreservation of bovine fibroblasts in straws after submission to negative pressure

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Keywords: controlled stress, cryopreservation, somatic cells.

The somatic cell cryopreservation plays an important role not only to aid in germoplasm preservation, but also in keeping genotypes used for experiments on induction of pluripotency, cell lineage formation and cloning by somatic cell nuclear transfer. The study of the effects of controlled stress in gametes and somatic cells has demonstrated that under certain experimental conditions, it can provide a higher survival rate, as well as it might play a role in parameters such as the growth pattern in culture. To assess that, bovine fibroblasts at first passage, obtained by standard procedures of cartilage explantation were randomly allocated into 4 experimental groups, as described: Fibroblasts were submitted (in culture) to 500 mBar of negative pressure during 4 minutes immediately (PN0h) or 3 hours (PN3h) before freezing (in standard procedures using 10% DMSO in D-MEM). After tripsinizing, cells were loaded into 0.25 mL straws, at a concentration of 1 x 106 cells/mL and 200 µL of total volume. The straws were sealed and stabilized in conventional fridge (5-7 ˚C) for 30 minutes, then exposed to nitrogen vapor (4 cm above the surface) for another 5 minutes and finally plunged. Thawing was performed in water-bath at 36°C for 20 seconds. Non-treated fibroblasts were used as fresh (FC) and cryopreserved (CC) controls. The parameters assessed were post-thaw viability, replication index (based in the cellular population doubling time - PDT), with intervals of 24 h and 8 days of length. At every session, one well of each group was tripsinized and the number of viable cells was estimated with the aid of haemocytometer after staining with trypan blue. The PDT was determined using the algorithm available online (http://www.doubling-time.com): TD = t x lg2/(lgNt – lgN0). Data were analyzed through ANOVA and T-test, with 5% of significance. The average cell survival rate of the groups CC (89.8%) and PN0h (88.1%) were higher than for the group PN3h (68.7%). The PDT length was similar among the groups FC (27.5±0.35 h); CC (30.1±2.3 h) and PN0h (32.4±1.6 h), with tendency (P=0.06) of an increase of PDT length for the group PN3h (34.8±2.3 h), in comparison to FC. Plastic straws were suitable for freezing bovine fibroblasts. No negative effects were observed after submitting the cells to negative pressure immediately before freezing. The culture for 3 h after the negative pressure reduces the growth rate of the cells, after freezing/thawing. New studies might be conducted, aiming to evaluate whether the treatment with negative pressure affects either the senescence pattern of the cells, or the viability of cloned embryos produced using negative pressure-treated somatic cells.

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A295 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Lipid dynamics in early bovine embryo development

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Keywords: early embryonic development, lipidomics, pre-implantation.

Mammalian pre-implantation embryonic development is a complex, conserved and well-orchestrated process involving dynamic molecular and structural changes. Understanding membrane lipid profile fluctuation during this crucial period is fundamental to address cellular and molecular mechanisms governing embryogenesis. Full understanding of stage-specific lipid signatures in early bovine embryo development is however still lacking. The primary aim of the present work was therefore to monitor the dynamic changes in stage-specific lipid profiles during early bovine embryonic development. For that purpose, MALDI-MS was used, and the observed changes were associated to the amount of cytoplasmic lipid droplets. Immature oocytes (-24 h post-insemination [hpi]) were recovered from slaughterhouse-derived ovaries, and 2-cell (32-40 hpi), 8 to 16-cell (72 hpi), morula (120 hpi) and blastocysts (168 hpi) were in vitro produced using two different culture media (SOFaaci supplemented with 2.5% of serum and serum-free SOF-BE1). For statistical analysis, uni- and multivariate models were used. Lipid content, monitored by lipid staining (n= 5-9 per stage), increased ($P<0.05$) at morula followed by a sharp drop ($P<0.05$) at blastocyst stage (58.4 ± 10.5^{ac}, 62.5 ± 9.4^{ac}, 85.9 ± 8.2^{a}, 148.3 ± 7.4^{b}, 37.4 ± 9.9^{c}; respectively for immature oocyte, 2-cells, 8 to 16-cells, morula and blastocyst). Cytoplasmic lipid droplets have increased ($P<0.05$) by SOFaaci at morula (162.6 ± 11.3 vs 137.1 ± 9.2) and blastocyst (49.9 ± 9.9 vs 20.7 ± 9.9) stages compared to SOF-BE1. Differences in the phospholipids profiles as monitored by MALDI-MS (n=5-9 per stage) were addressed by multivariate analysis. Characteristic dynamic changes were observed during early embryo development for the phospholipid profiles (phosphatidylcholines, sphingomyelins, and phosphatidylethanolamines), which were significantly related to unsaturation level, acyl chain length and class composition. This study provides a comprehensive analysis of stage-specific lipidome signatures in early bovine embryo development. The present data should be useful to assess the role of specific lipid species in important events of embryogenesis as well as to improve outcomes in both bovine and human assisted reproductive techniques.

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A296 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of seminal plasma addition of whole and vasectomized rams to semen after collection

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Keywords: addition, ram, seminal plasma.

The seminal plasma could have a protective effect when added to thawed semen. Little is known about its effect in addition to semen immediately after collection and if seminal plasma of high freezability semen collected from whole animals and vasectomized rams, can increase the cryopreserved semen of low semen freezability rams. The objective of this study was to evaluate the addition of seminal plasma collected from whole ram (INT) and vasectomized ram (VAS) of high freezability semen in semen of low freezability before the cryopreservation process. Four rams of low freezability semen were used as semen donors and two rams with high freezability semen as seminal plasma donors. Three treatments were used: VAS – Addition of 30% of seminal plasma from vasectomized ram in the middle of pre-dilution; INT – Addition of 30% of seminal plasma from whole ram in the middle of pre-dilution; and control (CONT) - without addition of seminal plasma. We evaluated the subjective motility, total motility and progressive motility by CASA and membrane integrity after thawing (TIME 0) and after the thermal resistance test (TRT) for 4 hours (TIME 4). For statistical analysis by calculating average and standard deviation of the parameters analyzed, followed by analysis of variance using the GLIMMIX procedures, and the comparison of averages made by Tukey test corrected for multiple comparisons. It used the SAS Enterprise 5.1 program. After thawed (TIME 0) the parameters subjective motility, total motility, progressive motility and membrane integrity in the VAS treatment (59.72 ± 3.4, 60.08 ± 2.6, 40.94 ± 3.4, 39.52 ± 2.7) and INT treatment (49.84 ± 2.9, 49.74 ± 3.6, 31.79 ± 3.4, 30.95 ± 2.6) was higher ($P<0.05$) than the CONT treatment (37.90 ± 2.5, 36.58 ± 3.5, 22.12 ± 1.9, 2.7 ± 21:18). With regard to the parameters after the TRT (TIME 4) subjective motility in VAS treatment (50.78 ± 3.6) and INT treatment (40.02 ± 4.0) was higher ($P <0.05$) than the CONT treatment (27.88 ± 2.6). As for the parameters of total motility, progressive motility and membrane integrity VAS treatment (51.92 ± 3.5, 35.46 ± 3.4, 38.78 ± 1.4) was higher ($P <0.05$) than INT treatment (38.86 ± 2.9, 24.36 ± 3.5, 24.11 ± 2.0) and CONT treatment (28.91 ± 2.2, 12.19 ± 3.0, 02.11 ± 1.6) and the INT treatment was higher ($P <0.05$) than CONT treatment. The addition of seminal plasma before the cryopreservation process, can improve the resistance of damage caused in cryopreserved ram semen, with seminal plasma collected from vasectomized animals having best results.

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A297 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of adding conjugated linoleic acid to *in vitro* culture medium in the viability after vitrified bovine embryo transfer

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Keywords: conjugated linoleic acid, embryo transfer, vitrification.

In vitro produced embryos exhibit low cryotolerance due to their high concentration of cytoplasmic lipids (Abe et al., J. Reprod. Dev., v.49, p.193, 2003). The conjugated linoleic acid trans-10, cis-12 (CLA) decrease lipogenesis in cells, improving embryo quality and possibly decreasing its sensitivity to cryopreservation (Mitchell and McLeod, Biochem. Cell Biol., v.86, p.293, 2008). It was evaluated the effect of adding CLA to in vitro culture medium in the viability after vitrified bovine embryo transfer. Three culture media were used: Control (n=340 oocytes): SOF medium added to BSA and FBS, without the addition of CLA; FBS+CLA (n=359 oocytes): SOF medium added to BSA, FBS and CLA; CLA (n=339 oocytes): SOF medium plus BSA added to CLA, without adding FBS. The blastocysts were submitted to the Open Pulled Straw vitrification method for subsequent heating. The recipient estrus was synchronized using protocol with intravaginal progesterone device (1.9g), associated with estradiol benzoate (2.0 mg) and estradiol cypionate (1mg), eCG (250UI) and analog PF2α (0.05 mg of sodium cloprostenol). There were transferred one or two embryos to the uterine horn ipsilateral to the corpus luteum (CL): T1 [recipients that received one blastocyst (n=17 embryos, Control=5, FBS+CLA=6 and CLA=6)]; T2 [recipients that received two blastocysts (n =54 embryos, Control=18, FBS+CLA=14 and CLA=22)]. In D7 after induced estrus the recipients were evaluated for CL confirmation. 58% of the animals responded with exacerbated form to estrus induction protocol, with multiple ovulations (2 to 7 CLs) and multifollicular growth (follicles up to 1.9cm and follicles larger than 2.0cm). The embryo transfer was performed only on animals with CL and follicles smaller than 1.9cm. To assess viability, vitrified embryos were heated (Control=27; FBS+CLA=30; CLA=17) and cultured in vitro to verify the reexpansion and hatching ability after 24, 48 and 72 hours of cultivation. There was no difference in blastocyst production relative to the total number of oocytes fertilization (37.1% for Control; 35.4% for FBS + CLA, CLA and 28.3%) ($P>0.05$). Only one pregnancy was observed in early and confirmatory diagnosis, result of a Control group embryo transfer. Pregnancy rates for Control group, FBS+CLA and CLA were, respectively, 4.35% (1/23), 0% (0/20) and 0% (0/28). Regarding the reexpansion, CLA treatment (47.1%) had lower rate ($P<0.05$) to Control (70.4%) and higher than the CLA+FBS (43.3%). The three treatments had similar hatching rates ($P>0.05$) by looking up 42.1%; 23.1%; 25% for Control; FBS+CLA; CLA groups, respectively. New embryo transfers should be carried out to evaluate the effect of CLA on in vivo embryo survival, endorsing the in vitro obtained reexpansion and hatching rates.

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Effect of handmade biopsy on bovine embryos at the sixth day post *in vitro* fertilization

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Keywords: biopsy, embryos, in vitro.

The genomic selection is a useful tool for animal breeding and it can be applied for in vitro produced bovine embryos through the analysis of embryo cells. However, it is necessary to evaluate the viability of the embryos biopsied at early stages. The aim of this work was to evaluate the impact of the biopsy technique without micromanipulation devices on development and further quality of embryos at the sixth day after in vitro fertilization. Oocytes obtained from ovaries collected at slaughterhouse were in vitro matured and fertilized. Followed in vitro fertilization, the presumptive zygotes were denuded and cultured in CR2aa medium supplemented with 2.5% SFB at 38.5ºC in an humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2. Embryos (morulas and early blastocysts) at the sixth day post-fertilization (D6) were randomly distributed in two groups: G1-control (n=61) and G2-biopsy (n=64). Embryos in drops of 20 L of TALP-HEPES with 2% SFB were sectioned by hand using microblades (Bioniche, Canada) under stereoscope microscope. After that, single embryos were cultured in drops of 20 L CR2aa. The blastocyst rate was evaluated on day seven (D7) and day eight (D8) post fertilization (24h and 48h after biopsy, respectively). On D8, the blastocysts from both groups were fixed and available by TUNEL assay (Promega, USA) for total cell number and number of apoptotic cells and then calculated the apoptotic index. The embryo development (blastocyst rate at D7 and D8) was analyzed by Qui-square. The total cell number, number of apoptotic cells and apoptotic index were analyzed by the t-Student test and their values are shown as mean +SEM. When only biopsied embryos at early blastocyst stage on D6 were analyzed, the blastocyst rate in G2 was lower ($P<0.01$) on D7 (43.5%) and on D8 (30.7%) than in G1 (78.7% and 67.8% on D7 and D8, respectively) but when the data of morula and blastocysts were grouped, the effect of biopsy on embryo viability was only perceived ($P<0.05$) on D8 (78.5% vs 43.5% for G1 and G2, respectively). There was no difference ($P>0.05$) in the total cell number between G1 and G2. However, the number of apoptotic cells was higher ($P<0.05$) in G1 than in G2 (29.4±4.5 vs 10.0±1.5, respectively). Similar finding was observed for the apoptotic index (27.5±3.9 vs 9.7±1.3, for G1 and G2, respectively, $P<0.01$). We concluded that the handmade biopsy in embryos on D6 influences the embryo development, but it doesn't affect the total cell number in blastocysts on D8, i.e, 48h post-biopsy. The lowest apoptotic index found in biopsied embryos may be due to the unnoticed withdrawal of cells from the inner cell mass during the biopsy, since this site display great number of apoptotic cells.

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A299 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of protein source during culture on *in vitro* bovine embryo resistance to cryopreservation by slow freezing

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Keywords: BSA, classic freezing, feasibility.

The culture conditions can influence directly the quality of IVP embryos and their sensitivity to cryopreservation. Supplementation of IVM and IVC media with FBS is routinely used in IVP, however the presence of serum may result in accumulation of lipids and reduction in cry tolerance. BSA is an alternative source of protein that can be used to replace FBS that can improve the response of IVP embryos to cryopreservation (HIROYUKI ABE. MOLECULAR REPRODUCTION AND DEVELOPMENT.61:57-66.2002). The objective of this study was to evaluate the effect of these different protein sources on embryonic development and resistance to cryopreservation by slow freezing of bovine embryos. COCs obtained from slaughterhouse ovaries were selected and distributed into 2 groups: 1) control: COCs matured and cultured in media supplemented with 10 and 5% FBS, respectively; 2) COCs matured and cultured in media supplemented with 0.4% BSA. In D7, embryos at the BX stage from each group were divided into two treatments, half was kept fresh and the other half was frozen. The freezing was done using EG 1.5M in 0.25 ml straws and placed in Freezing Machine(FREEZE CONTROL® ,Model CL-863 System, Cryologic, Australia). After thawing the embryos were evaluated at 24 and 48 hours for reexpansion and hatching rates. Data were analyzed by chi-square test ($P < 0.05$). Cleavage rate was similar between group 1 (85.8%, n = 239) and 2 (81.7%, n=213), but the blastocyst rate was highest in group 1 (41.4%, n = 99 vs 26.8%, n = 57After thawing, the group cultured in the presence of FBS and cryopreserved, showed a lower hatching rate at 24 h (3.2%) than the fresh group (30.6%). While in the group cultured with BSA the hatching rate was similar between fresh (19.0%) and cryopreserved (13%) embryos. At 48 h of post thawing culture the hatching rate was lower in frozen than fresh embryos from both groups with FBS (6.5 and 61.1%) and with BSA (30.4 and 61.9%). However, for the cryopreserved embryos, a higher hatching rate was observed for those cultured in the presence of BSA than those in the presence of FBS. The results suggest that embryos produced in the absence of FBS although have lower blastocysts production have better resistance to freezing than those in the presence of FBS.

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A300 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of supplementation with linoleic acid (omega 6) during *in vitro* production of bovine embryos on the modulation of intracytoplasmic lipid content and resistance to cryopreservation

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Keywords: bovine embryo, cryotolerance, linoleic acid.

This study aimed to evaluate the effects of the addition of linoleic acid (LA) to the culture medium in different moments of in vitro production (IVM, IVC or IVM+IVC) on the accumulation of intracytoplasmic lipids, development and cryotolerance of bovine embryos. COC's (n=526) were matured in TCM 199 with 25 mM bicarbonate, 10% FCS, 0.5 µg/mL FSH and 100 IU/mL hCG, supplemented or not with 100 mM LA, without mineral oil, for 22h at 38.5°C and 5% CO_2 in air. After, the oocytes were subjected to IVF and presumptive zygotes were cultured in SOFaa supplemented or not with 100 mM LA, without mineral oil, at 38.5°C and 5% CO_2 in air. Thus, there were four treatments: Control, LA during maturation (IVM), LA during embryo culture (IVC), and LA during IVM and IVC (IVM+IVC). The embryo development was evaluated on D7, when expanded blastocysts (Bx) were stained or cryopreserved. For determination of intracytoplasmic lipid content, a sample of Bx (n=133) was stained with the lipophilic dye Sudan Black B and stained embryos were evaluated under a light microscope running Q-Capture Pro Image software. The images were converted to grayscale and embryos were delimited to determine the lipid content. The Control group was chosen as the calibrator and the gray intensity value of each group was divided by the average of the calibrator to generate the values in arbitrary units. Other Bx (n=68) were vitrified by Vitri Ingá® (Maringa, PR, Brazil) method. The rates of re-expansion were evaluated immediately after warming (0h) and after additional 24h of IVC in SOFaa medium. The embryonic development rates and re-expansion rates were evaluated by Chi-Square, while the lipid content was subjected to analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$). There was no significant difference ($P>0.05$) in the blastocyst rates between the Control (46.7%) and IVM (42.9%) and IVC groups (46.6%), but embryo production was lower ($P<0.05$) in IVM+IVC group (32.8%) compared to Control and IVC groups. The intracytoplasmic lipid content in embryos of the Control group (1.0±0.0) was similar ($P>0.05$) to that of the IVM group (0.9±0.1), but differed ($P<0.05$) from IVC (08±0.1) and IVM+IVC groups (0.7±0.1). Based on these results, we decided to evaluate the cryotolerance of embryos treated with LA only during IVC, and we observed that although no significant difference was found ($P>0.05$), the rates of re-expansion after 24h were numerically higher in IVC group (28/43; 65.1%) when compared to Control (18/39, 46.52%). Nevertheless, the biological effect was evident, since a greater number of transferable structures were retrieved. In conclusion, treatment with LA reduced the amount of intracytoplasmic lipids in bovine IVP embryos, which resulted in the recovery of a greater number of viable blastocyst after warming, but this difference was not statistically significant.

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A301 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of two FSH ovarian stimulation treatments on doppler velocimetry and ovarian artery diameter in goats

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Keywords: color Doppler, goats, ovarian artery.

The aim of this study was to evaluate the effect of two FSH ovarian stimulation treatments on Doppler velocimetry and diameter of ovarian artery. 4 undefined breed goats 4-7 years of age were used. For synchronization of the cycle, intravaginal sponges were inserted containing 60 mg of MAP for 10 days, on the seventh day of treatment 50 ug D-cloprostenol was applied. For ovarian stimulation, the goats were divided into two groups; i) multi-dose (MD) with administration of 120 mg of pFSH intramuscularly, divided into five decreasing doses. Progestogen was administered on the 7th day of the treatment till 24 hours before sponge removal in 12 h intervals; ii) one-shot (OS) pFSH with 70 mg plus 200 IU eCG administered intramuscularly 36 hours before withdrawal of the sponge. Doppler ultrasound scanner apparatus (CTS-8800V, SIUI, China) equipped with linear transrectal probe (6-8 MHz) was used. Goats were maintained in stationed position and urinary bladder was used as a guide, the probe was inserted with the transducer perpendicular to the ventral abdominal wall and scanning was performed at 90° clockwise and 180° counterclockwise for location of the left and right ovaries, ovarian arteries and uterine veins. The examinations were performed before insertion and after removal of sponge to measure the diameter of vascularization in ovarian artery. The angle of insonation was set at 60, and the images were analyzed using the program Image J (National Institutes of Health, USA) previously calibrated (62.3 pixels/cm). Data was presented as mean ± standard deviation and paired-t test was used to assess differences between groups. The visualization of blood flow in ovarian artery in all ultrasound examinations was possible. The End Diastolic Flow (EDF) in the MD and OS group on D0 was 11.20± 1.94 and 13.5± 3.91, respectively. The average EDF on D10 in MD and OS groups was 15.3± 3.91 and 15.5± 5.17 cm/sec, respectively. The average Peak Systolic Velocity (PSV) in MD and OS groups on D0 was 25.6 ± 7.60 and 4.6± 28.5 cm/s, respectively, the same parameter on D10 averaged 27.4± 3.71 for MD group and 29.7± 6.08 cm/s for OS group, with no differences between groups in Doppler parameters ($P < 0.05$). The diameter on D0 of the ovarian artery in MD and OS groups was 0.21 ± 0.03 and 00.7 ± 0.17, respectively, whereas at D10, the averages were 0.30 ± 0.04 and 0.22 ± 0.03 in MD and OS groups, respectively. Significant differences ($P > 0.05$) were observed between days D0 and D10 in the MD group, but there was no difference between groups on D10. In conclusion, the use of two different treatments for ovarian stimulation did not alter the blood irrigation in caprine ovary.

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A302 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of Fibroblast Growth Factor-10 during pre-IVM and IVM on quality of IVP bovine embryos

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Keywords: embryos, gene expression, oocyte competence.

Aiming to improve oocyte competence, a pre-IVM period in which the resumption of meiosis is blocked immediately after the oocyte retrieval has been proposed by several authors. However, no increase on embryos production has been obtained to date (Guemra et al., 2014, Theriogenology, v. 81, p. 982–7; Guimarães et al., 2015 Theriogenology, 83:52-7). Fibroblast growth factors (FGF) have an important role from since the beginning of oocyte formation until atresia or ovulation. Among the FGF family, addition of FGF-10 to the medium during IVM improves embryo quality (Zhang et al, 2010, Reproduction, v. 140, p. 815–26). This study aimed to evaluate if supplementation of the pre-IVM and IVM medium with FGF10 would improve the quality of bovine embryos assessed by the kinetics of development and the gene expression. COCs were aspirated from slaughterhouse ovaries and after selection, were divided into 3 groups: T1 (control), with COCs matured for 22 hours (N = 116); T2 (PMC-MC), with COCs pre-matured for 22 hours and matured for 22 hours (N = 119); T3 (PFGF-MFGF) with COCs pre-matured with FGF10 for 22h and matured for 22 hours (N = 112). The pre-IVM medium contained 0.2% BSA, 10-4UI / ml FSHrh and 10μM of cilostamide, and the IVM medium, 0.4% BSA and 10-1UI / ml FSHrh. The FGF10 concentration was 0.5 ng/ml. After maturation, COCs were fertilized and cultured until D8 of development. Hatched blastocysts on D8 were frozen in RNA Later for gene expression analysis by qPCR. Blastocyst rate was evaluated on D6, D7 and D8 and embryos were categorized into Bi, Bl, Bx and BE. The relative expression of KRT8, PAGE2, PLAC8, MSH6, HSPB1 genes, was assessed by qPCR using Fast Sybr Green Master Mix (Applied Biosystems). The ACTB gene was used as endogenous control. Data of blastocyst rate and kinetics of development were evaluated by chi-square test. The relative expression of each gene was calculated using the ΔΔCt method with efficiency correction by Pfaffl method, the treatments were compared using the Dunnett test ($P < 0.05$). There was no difference between treatments in the blastocyst rate at D6 and D7 (T1: 33.6; 53.4; T2: 28.6; 43.7; T3: 33.9; 47.3). On D8, T2 had lower blastocyst rate than the other groups. The addition of FGF-10 did not increase the rate of embryo development, since Bx rate on D7 and Be rate on D8 (T3: 64.2 and 72.0) was similar to T2 (50 and 64.6) and T1 (77.4 and 56.2). From all genes evaluated only MSH6, which is related to DNA repair, showed difference in expression between treatments, being lower in T3 group compared to the control ($P < 0.05$). The results suggest that the addition of FGF10 during pre-IVM and IVM does not affect the quantity or quality of bovine IVP embryos.

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A303 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effects of Forskolin during *in vitro* culture on the modulation of intracytoplasmic lipid content and resistance of bovine embryos to cryopreservation

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Keywords: cryotolerance, Forskolin, in vitro bovine embryos production.

This study aimed to evaluate the effects of different concentrations of Forskolin, added to the *in vitro* culture medium, on the intracytoplasmic lipid content, development and cryotolerance of bovine embryos. COC's (n=848) were submitted IVM during 22h at 38.5°C and 5% CO_2 in air, in microdroplets of TCM 199 with 25 mM of bicarbonate, 10% FCS, 0.5 µg/mL FSH and 100 IU/mL hCG, covered with mineral oil. After, the oocytes were subjected to IVF and the presumptive zygotes were cultured in SOFaa (control Group), supplemented on D6 with 2.5 (F2.5 Group), 5.0 (F5 Group) or 10 µM of Forskolin (F10 Group), at 38.5°C and 5% CO_2 in air. The embryo development was evaluated on D7, when expanded blastocysts were stained or cryopreserved. For the determination of intracytoplasmic lipid content, a sample of expanded blastocysts (n=99) was stained with the lipophilic dye Sudan Black B and embryos were evaluated under a light microscope running Q-Capture Pro Image Software. The images were converted to grayscale and embryos were delimited for determination of lipid content. The control group was chosen as the calibrator and the gray intensity value of each group was divided by the average of the calibrator to generate the values in arbitrary units. Other expanded blastocysts (n=76) were vitrified by Vitri Ingá® - Maringa - PR - Brazil method. The re-expansion rates were evaluated immediately after devitrification (0h) and after 24 hours of in vitro culture in SOFaa medium. The embryo development rate and re-expansion were evaluated by Chi-Square test, while the lipid content was subjected to analysis of variance (ANOVA) followed by Tukey's test ($P<0.05$). There were no significant differences ($P>0.05$) in the blastocyst rates between the Control group (44.9%) and other treatments, but embryo production was lower ($P<0.05$) in F10 group (38.8%) compared to groups F2.5 (50.5%) and F5 (54.7%). The amount of intracytoplasmic lipids of the Control group (1.0±0.00) was similar ($P>0.05$) to F2.5 (0.9±0.03) and F10 groups (1.06±0.03), however it differed ($P<0.05$) from F5 group (0.8 ±0.1). Based on these results, F5 group was tested for cryotolerance and it was observed that the rate of re-expansion 24h was greater ($P<0.05$) in F5 group (72.2%) compared to the Control group (46.2%). In conclusion, treatment with Forskolin at a concentration of 5 µM is effective for reducing the amount of intracytoplasmic lipids and improves cryotolerance of IVP bovine embryos.

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A304 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of cAMP modulators during oocyte *in vitro* maturation on nuclear maturation and cytoskeleton integrity of vitrified bovine oocytes

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Keywords: cilostamide, Forskolin, IBMX.

Cryopreservation of oocytes is a strategic tool in embryo IVP but with limited use due to the complex cellular structure of oocytes, being oocyte quality a factor that influences the success of the technique. In view of the role of IVM on oocyte quality, Simulated Physiological System Oocyte Maturation (SPOM; Albuz, Hum Reprod, vol 25, p 12; 2010), which utilizes cAMP modulators to achieve greater oocyte competence by the extension of meiosis block, was developed. The aim of this study was to investigate the effect of SPOM system on nuclear maturation and cytoskeletal integrity of vitrified bovine oocytes. Oocytes from slaughterhouse ovaries were divided into 8 groups: G1 (immature oocytes); G2 (matured in standard medium without FCS / 24 h); (G3 subjected to pre-IVM / 2 h in the presence of modulators of cAMP, Forskolin (100μM) and IBMX (500μM), and then the extended IVM / 28 h with Cilostamide (20μM) and FSH); G4 (immature oocytes vitrified and subjected to conventional IVM); G5 (immature vitrified and subjected to pre-IVM and extended IVM); G6 (submitted to pre-IVM, vitrified, and then the extended IVM); G7 (matured in commercial IVM - Bioklone ® Animal Reproduction, São Paulo, Brazil - with SFB / 24 h); G8 (immature vitrified and subject to commercial IVM, with SFB). For analysis of nuclear maturation, oocytes (n = 429 obtained in 3 replicates, 15 to 75 per experimental group) were stained with Hoechst 33324 for obtaining the rate of matured oocytes (MII). Cytoskeletal actin filaments were stained with Phalloidin Atto-532 to evaluate the actin staining patterns (Stained / Uninjured or not stained / Injured; n = 373 obtained in 2 replicates, 9 to 73 per experimental group). The M II rate was evaluated by chi-square test ($\chi 2$) and the percentage of staining patterns/integrity of actin by Fisher's exact test, in Instat GraphPad program, the significance level of 5%. The MII rate observed in groups was: G1 (Immature): 6.67 ^{a}; G2 (Standard IVM): 77.78 ^{c}; G3 (SPOM IVM): 76.19 ^{c}; G4 (VIT/Standard IVM): 31.43 ^{ab}; G5 (VIT/pré-IVM/extended IVM): 18.57 ^{a}; G6 (pre-IVM/VIT/extended IVM):): 39.47 ^{b}; G7 (Commercial IVM): 71,74 ^{c}; G8 (VIT/Commercial IVM): 25.71 ^{ab}. This result suggests that in vitrified groups maturation was impaired, but to a lesser extent in the group undergoing pre-MIV before vitrification. Regarding the pattern of actin (Uninjured / Injured), we observed: G1: 100.0^{a} / 0.0; G2: 100.0^{a} / 0.0; G3: 100.0^{a}/0.0; G4: 50.0^{bd} / 50.0; G5: 26.0 ^{c} / 74.; G6:60.3 ^{b} / 39.7; G7: 94,2^{a} / 5.8; G8: 37.9 ^{cd} / 62.1. Therefore, our results suggest that in vitrified groups the injured integrity pattern is predominant, except in the group undergoing pre-MIV before vitrification. The pre-IVM SPOM system favors meiotic progression and cytoskeletal integrity of oocytes undergoing vitrification, and its use can improve rates at oocyte cryopreservation programs.

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A305 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Interference of cGMP pathway on the expression of lipid metabolism transcripts in bovine cumulus-oocyte complexes matured *in vitro*

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Keywords: nucleotides, PDE5, sildenafil.

In adipocytes, lipolysis depends on the activity of cAMP or cGMP-dependent protein kinase (PKG), therefore, intracellular levels of cAMP and cGMP nucleotides regulate lipolysis; when elevated it is increased and when reduced lead to lipogenesis (LAFONTAN, Endocrinololy, 19 (4): 130-7.2008). Enzymes controlling cAMP and cGMP levels (synthesis and hydrolysis) or activated by them are expressed in oocytes (OO) and cumulus cells (CC) (Schwarz, Theriogenology, 81 (4): 556-64.2013), therefore, it is possible that manipulating these signaling pathways during maturation may influence lipid metabolism in cattle cumulus-oocyte complexes (COCs). The aim of this study was to investigate cyclic GMP pathway during oocyte maturation and evaluate its effect on the transcription of genes involved in lipid metabolism. Two experiments were conducted in which COCs were matured in TCM199 with 0.4% BSA or 10% FBS plus 10-5 M sildenafil (SDF- inhibitor of PDE5, the cGMP degrading enzyme) FBS+SDF+KT5823 (PKG inhibitor) for 22h. The control group matured with BSA only. After IVM, cGMP levels were measured (pool of 40 COC) using an immunoassay kit (Direct Cyclic GMP EIA) in experiment 1. In experiment 2, ATGL and PLIN2 (lipolysis related proteins) and CPT1B (beta-oxidation related protein) transcripts were assessed by real-time PCR in CC and OO from a pool of 20 COCs. Statistical analysis was performed by ANOVA followed by Tukey hoc test (SAS Institute Inc., Cary, NC, USA). The cGMP measurement data (three replicates), were transformed to log10 prior to analysis and for gene expression the values of $2^{-\Delta\Delta Ct}$ were considered (5 replicates). The SDF in groups matured COCs with BSA or FBS (56.84 and 53.08 fmol/pool) increased cGMP relative to control (38.07 fmol / pool, P>0.05) while SFB alone had the lowest values (23.08fMol / pool, P<0.05), but there was only difference between the group associated with KT5823 (61.47fMol / pool) in relation to that matured only with FBS (P <0.05). The three studied genes were expressed in both cell types, and ATGL has not yet been described in COCs. In CC, ATGL relative expression of SDF was reduced compared to the BSA and FBS (P0.05) and restored when associated with KT5823 (P<0.05) and CPT1B was elevated only with SDF+KT5823 (P<0.05). Regarding PLIN2, expression was increased by FCS compared to control BSA (P<0.05), but adding SDF returned to control levels and association with KT5823 increased expression (P<0.05), similar to FBS. In OO there was no effect of treatments (P<0.05). In conclusion: 1) PDE5 and PKG are involved in the control of cGMP levels in bovine COCs and SFB tends to reduce such levels; 2) the pathway studied influences the expression of genes involved in lipid metabolism in CC, which in turn may have an effect on lipid metabolism of oocytes.

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A306 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Isolation of somatic cell derived from ear tissue of collared peccary (*Pecari tajacu linnaeus*, 1758) submitted to different vitrification techniques

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UFERSA.

Keywords: cryopreservation, somatic cell, wild animals.

The cryopreservation of tissue samples from wildlife species, especially collared peccary (*Pecari tajacu*), is an interesting step in obtainment and conservation of somatic cells for use in nuclear transfer (cloning). However, tissue vitrification protocols need to be optimized to ensure maximum preservation of the viable characteristics of cells. Therefore, the aim of this study was to compare by *in vitro* culture (IVC) two ear tissue cryopreservation techniques [conventional vitrification directly, CVD, as Silvestre *et al*. (2004, Theriogenology 49, 221–229) or solid-surface vitrification, SSV, according to Carvalho *et al*. (2011, Theriogenology 76, 933–941)]. Thus, ear fragments (9.0 mm^3) of eight collared peccaries (4 males and 4 females) with 7-12 months old, from Center Multiplication of Wild Animals (CEMAS/UFERSA), were collected and cryopreserved in solution containing Dulbecco Modified Eagles Medium (DMEM) plus 3.0 M dimethylsulfoxide, 3.0 M ethylene glycol, 0.25 M sucrose and 10% fetal bovine serum. After two weeks, fragments warmed and not cryopreserved (control) were cultured *in vitro* and assessed for cell morphology, adhesion, early subconfluence (70% of the plate covered by cells) and cell viability by trypan blue (in %). All data from eight animals were analyzed by ANOVA followed by the appropriate *post-hoc* test. After collection of tissue samples, a total of 96 fragments were distributed for the three experimental groups (CVD: 32; SSV: 32 and control: 32 fragments). Of these, only one fragment of control group did not promote adherence after the IVC. All fragments adhered in all groups were able to cell confluence, with cell proliferation from days 7°, 5° and 3° for the CVD, SSV and control groups, respectively. Differences in initiation of proliferation were observed between the cryopreserved and control groups ($P<0.05$). In general, all cells showed fusiform features with oval nucleus in the center, suitable fibroblasts. Moreover, all the adhered samples were able to achieve subconfluence on days 17.5°; 18.3° and 18.0° to the CVD groups, SSV and control, respectively ($P>0.05$). No difference was observed for the viability of growing cells (CVD: 89% *vs.* SSV: 86% *vs.* control: 91%, $P>0.05$). Additionally, whereas the control group reached as maximum viability, cells derived from the CVD and SSV groups showed a viability of 98% and 95%, respectively. In conclusion, both vitrification techniques may be used for the cryopreservation of somatic tissue of collared peccary, allowing the isolation of viable cells for the cloning and genetic conservation of this species.

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A307 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Global methylation and hydroxymethylation patterns of bovine tissues

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Keywords: *Bos taurus indicus*, hydroxymethylation, methylation.

The ratio of 5-mC to 5-hmC seems to have an important role in epigenetic reprogramming during gametogenesis and initial embryogenesis, involved in gene expression regulation. The aim of this study was characterize the global patterns of DNA methylation and hydroxymethylation in *Bos taurus indicus*. Fetal tissues of newborn Nellore calves and Gyr bull's spermatozoa from epididymis (head an tail) or from ejaculate, were used as DNA source. Blood samples (n=19) e placental tissues (cotyledon and allantoidal membrane; n=12) were collected soon after birth, stored at -20ºC and DNA was extracted using the *QIAamp DNA Blood Mini* kit (QIAGEN, Hilden, Germany). Ejaculated spermatozoa were obtained through eletroejaculation (n=5) and spermatozoa of epididymis head (n=5) and tail (n=2) were obtained using the extravasation method. The DNA extraction was procedure using a *Salting Out* protocol. Global DNA methylation and hydroxymethylation were quantified using the *MethylFash Methylated* and *MethylFash Hydroxymethylated DNA Quantification* (Epigentek, NY, USA) kits, respectively. DNA methylation means in blood, allantoidal membrane and cotyledon were 12.68±6.75%, 17.26±15.07% and 12.94±5.49%, respectively. DNA hydroxymethylation means were 3.47±1.93%, 2.86±3.16% and 2.43±2.12%, respectively. It was not observed any difference between the evaluated tissues, but a difference in hydroxymethylation was observed in cotyledon between male and female (4.15±1.67% and 0.99±1.13%, respectively). DNA methylation means in spermatozoa of epididymis head and tail and ejaculated spermatozoa were 43.27±6.84%, 34.66±8.04% and 35.12±6.40%, respectively; the means of DNA hydroxymethylation were 13.69±7.22%, 6.71±3.03% and 14.34±2.79%, respectively. The 5-mC and 5-hmC patterns do not alter during the maturation and post-ejaculation processes. However, in spermatic cells, the methylation and hydroxymethylation levels were higher than in blood and placental tissues; an exception is the 5-hmC in epididymis tail spermatozoa, which did not present any difference to the other evaluated tissues. 5-mC/5-hmC ratios for blood, allantoidal membrane, cotyledon, and spermatozoa from epididymis head and tail and from ejaculate were 4.75±3.77; 10.41±10.13; 8.36±8.27; 4.31±2.91; 5.44±1.25 e 2.53±0.65, respectively, and they did not alter in different studied tissues. The results showed that 5-mC and 5-hmC levels alter among the different tissues evaluated and according the gender in extra-embryonic tissues. It is important to emphasize the pioneering of this work in bovine and the relevance of this knowledge to improve our comprehension about the epigenetic mechanisms.

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A308 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Short-term preservation of bovine COC in rainy season of Araguaína-TO: effects of temperature and time

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Keywords: bovine, conservation, COC.

Recent research has created extraordinary opportunities for animal reproduction, provided a major revolution in the multiplication of high economic potential animals. Biotechniques have been developed to optimize and support the other already established, such as IVF and MOIFOPA. Therefore, this study aims to evaluate the effect of time and transport of slaughtered bovine ovaries temperature on the Cumulus Complex - oophorus (COC) and analyze its viability after this procedure. For this purpose were collected 30 pairs of ovaries in the municipal slaughterhouse Araguaína, in rainy season, transported in thermos bottles containing 0.9% saline solution. For analysis, the ovaries were divided into six experimental groups, according to temperature and time each was analyze. The time intervals tested were 3, 12 and 24 hours at temperatures of 4°C and 37°C. Antral follicles were aspirated to obtain COC with syringe needles coupled to 25x7 or 25x8. After the follicular aspiration, COC obtained were stained with 4% Trypan Blue (1: 1) to verify the integrity of the plasma membrane of granulosa cells and / or oocyte. Descriptive statistics were performed and the results were expressed in percentage (Andrade et al. J. Small Rumin. Res. V. 43, p. 235, 2002). COC 281 bovine were evaluated, in which the ovaries were transported within three hours, at both temperatures, they do not have their COC stained with Trypan Blue, suggesting no interference with viability. However, the ovaries were transported for a period of 12 hours, they started to show a decline in viability of COC and when exposed to different temperatures, those transported at 4°C showed better retention rate (65%) when compared having a temperature of 37°C, which obtained a relatively low rate of viable COC (46%), suggesting damage to the plasma membrane of these cells. When tested 24 hours, ovaries, transported at 4°C presented with normal aspect, although only 13 COC were obtained, with 77% viability. Already the ovaries transported in 37ºC in that same period, were damaged and difficult aspiration, and showed 100% COC unviable. From these results, it was concluded that there is a decline in viability of bovine COC when the ovaries were transported in saline at 4°C or 37°C, for periods over 12 hours, during rainy season.

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A309 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Bovine embryo production by metaphase plate transfer

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Keywords: metaphase plate, oocyte competence, oocytes.

The metaphase plate transfer (MPT) between oocytes provides the ability to solve problems that can compromise the success of some assisted reproductive techniques. Among those, the age of the donor, the presence of abnormalities caused by mutation of the mtDNA and cytoplasmic damage due to cryopreservation can be mentioned. In addition, this procedure is a tool to study various aspects of the nuclear-cytoplasmic interaction in oocytes. The objective of this study was to evaluate embryo production from MII oocytes that had metaphase plate transferred to the cytoplasm of another enucleated oocyte. Cumulus oocyte complex (COC's) grades 1 and 2 obtained from slaughterhouse ovaries were matured for 20 hours at 38.5°C and 5% CO_2. After maturation, the COC's were denuded in 0.2% hyaluronidase solution and selected for the presence of polar body (PB) and cytoplasm homogeneity. The selected oocytes were divided into two groups: 1) control group: submitted to parthenogenetic activation (PA); 2) oocytes undergoing MPT and PA. Oocytes of group 2 were incubated in Cytochalasin D and Hoechst 33342 solutions in TCM-199, for 30 minutes. After incubation oocytes were enucleated with an aspiration pipette (diameter: 30 to 40 μm) coupled to a micromanipulator and immediately received a metaphase plate previously removed from a donor oocyte in MII stage. At the end of reconstruction, the structures were submitted to fusion (two pulses of 2.1 KV / cm). The fused structures (n = 42; 56.05% from total) and control oocytes (n = 42) were activated in Ionomycin 5 μM solution for 5 minutes and then maintained at 2 mM 6-DMAP solution for 4 hours. Subsequently, they were cultured for 7 days on cumulus cell (CC) monolayer at 38.5 °C and 5% CO_2. Presumptive zygotes were evaluated at D2 and D7 to cleavage and blastocyst rate, respectively. The results were analyzed by chi-square test with $P \leq 0.05$. The cleavage and blastocyst rates of group 2 (19.05% and 4.8%, respectively) were lower than the control group (90.48% and 47.6%, respectively). According to the results, we can conclude that MPT is a promising strategy to produce embryos from compromised competence oocytes due to freezing, by mtDNA alterations and/or other reasons.

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A310 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Embryo response from Piau and Moura porcine breeds to cryotop vitrification

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Keywords: embryo, porcine, vitrification.

The porcine breeds Piau and Moura are locally adapted and are being replaced by commercial breeds, which have increased the danger of being extinct. Therefore, there is a demand for conservation of genetic material of these animals. However, the cryopreservation of pig embryos has been limited by their high cryosensitivity. Vitrification and, specifically the Cryotop method, have been proposed as a more successful methodology for embryo cryopreservation for many mammalian species. This procedure has the advantages of no ice crystals formation and high cooling speed. In this study, we evaluated the response of pig embryos from Piau and Moura breeds to vitrification by Cryotop. Piau (n = 58) and Moura (n = 18) females had oestrus observed and on D6 after natural mating, in vivo embryo collections were performed by laparoscopic. A total of 353 embryos was recovered (108 Moura and 245 Piau) in different stages of development. For vitrification, only fresh embryos at BL and BX stages, grades I and II, were used. Embryos were distributed into control group (C) – not criopreserved, and vitrified group (V) for each breed. After the warming period, the embryos from both groups were cultured in SOF medium, at 38.5°C and 5% CO_2 in air. At 24 and 48 h of culture, re-expansion and hatching rates were evaluated. Hatched embryos at 48 h were frozen and stored for gene expression quantification by qPCR, in which SYBR Fast Green Master Mix was used. Data from re-expansion and hatching rates were analyzed by Chi-square test ($P< 0.05$) and the expression of genes was performed by $\Delta\Delta Ct$, corrected by Pfaffl, using the ACTB as an endogenous control. At 24 h of culture hatching rate was lower ($P<0.05$) in Piau embryos (57.4%) than in Moura embryos (83.9%), while for the vitrified hatching rate was similar ($P>0.05$) between Piau (8.9%) and Moura (0%). At 48 h of culture the hatching rate was similar ($P>0.05$) for the control embryos of both breeds (83.0% and 96.8%, respectively). The vitrified embryos of Piau and Moura breeds had similar ($P>0.05$) hatching rates (19.6% and 21.9%, respectively), but both presented lower ($P<0.05$) hatching rate than the control embryos. For gene expression analysis were used three embryos pools, C and V for Piau breed. Moura embryos were unable to perform qPCR, due to insufficient number. Among the analyzed genes, BCL2L1, BAK, CASP3, only BAX showed differential expression between groups, being more abundantly expressed for group V ($P = 0.04$) compared to C. The results indicate that porcine embryos, even vitrified by Cryotop, have low recovery after cryopreservation. However, the gene expression response suggests that embryos that hatched at 48 h reverted well from vitrification stress.

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A311 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Use of ethyleneglycol monomethyl ether as cryoprotectant in vitrification of IVP bovine embryos

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Keywords: cryoprotectants, ethylene glycol monomethyl ether, vitrification.

Research has been conducted to identify cryopreservation protocols using satisfactory vitrification process. However, little attention was given to the thermodynamic and chemical characteristics of cryoprotectants. The aim of this study was to determine the most effective concentration of ethylene glycol monomethyl ether cryoprotectant (EGMME) in the vitrification solution of in vitro produced (IVP) bovine embryos. This experiment determined the concentration of EGMME in the vitrification solution associated with better hatching rate after warming by measuring hatching rates and gene expression of 405 embryos, in 6 repetitions. The vitrification methodology was described previously by Vieira et al. (Animal Reproduction Science, v. 99, p. 377-383, 2007). On average it was used 22.5 embryos per treatment/repetition. Embryo hatching rate was obtained from the average value of each treatment; 30 warming embryos were transfer to synchronized recipients. The hatching rate of non-vitrified control group (63.8%) was higher ($p<0.05$) in comparison with the treatment of 20% EG and 20% DMSO (T2; 37.6%) and EGMME 20% DMSO and 20% (T3; 22.0%), which was similar ($p>0.05$) between each other. The hatching rate observed in the treatment EGMME containing 15% DMSO and 20% (T4; 10.3%) was lower ($p<0.05$) when compared with other groups. The gene expression of BAX (apoptosis promoter) and CCND2 (proliferation marker) did not differ ($p>0.05$) between groups, but the expression of Bcl-2 gene (inhibitor of apoptosis) was lower ($p<0.05$) in T4 compared with other treatments. Pregnancy rates at 30 days for T2 and T3 groups were both 26.6%. The embryos in T4 were not transferred to recipients. Therefore, EGMME can be used as cryoprotectant in vitrification solutions of IVP bovine embryos.

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A312 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Use of microfluidic in the analysis of profile of genes related to embryo quality from Nelore cows submitted to superstimulation with p36 or p36/eCG protocols

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Keywords: gene expression, microfluidic, ovarian superstimulation.

Superstimulatory protocols with exogenous gonadotropins have been widely used in bovine embryo technologies. Better understanding of follicular dynamics has enabled the development of hormonal protocols to boost fertility in cows, e.g. the P36 superstimulatory protocol. Studies have demonstrated that P36 adaptations, such as the replacement of the last two doses of FSH by eCG (P36/eCG protocol) improves embryo yield, possibly due to its capacity to stimulate both FSH and LH receptors, especially to FSH receptor. However, the quality of the embryos produced under the influence of these gonadotropins is conflicting, i.e. sometimes benefits and sometimes impairs embryonic development. Therefore, the present study aims to quantify the mRNA abundance of genes related to embryo quality in cows submitted to P36 and P36/eCG protocol. Nelore cows (Bos taurus indicus) were submitted to cycle synchronization (control group, n=20; non-superstimulated), P36 protocol (P36 group, n=15) and P36/eCG protocol (P36/eCG group, n=20). All animals were slaughtered 12 h after removal of progesterone device. Ovarian antral follicles from control group (3-8 mm) and superstimulated groups (> 9 mm) were aspirated for in vitro production of embryos. Total RNA was extracted from pools of 5 embryos (5, 3 and 5 pools for control, P36 and P36/eCG groups, respectively), followed by reverse transcription and pre-amplification in 14 cycles with Taqman® enzyme and Taqman® bovine-specific oligonucleotide primers. For quantification of the mRNA abundance of 46 genes related to embryo quality by RT-qPCR, we used the microfluidic platform BioMark HD System™ (Fluidigm®) with HX controller (96 samples and 96 assays at the same time, 96.96 Dynamic Array). The relative abundance was calculated by ΔΔCt method (target gene/GAPDH) with correction by Pffafl's equation. Superstimulatory effect was tested by ANOVA (parametric) or Wilcoxon test (non-parametric, statistical difference with $P < 0.05$). The mRNA abundance of IFITM3 and SOX2 was higher in blastocysts from control group compared to cows submitted to ovarian superstimulation, while the mRNA abundance of ACTB, AQP3, CASP3, CDH1, OTX2 and PAF1 was higher in blastocysts from superstimulated cows. Thus, the present findings demonstrate that the use of exogenous gonadotropins modifies the profile of mRNA abundance of genes related to embryo quality and it, therefore, might affect the competence of embryos generated from cows submitted to ovarian superstimulation.

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A313 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Vitrification of immature bovine cumulus-oocyte complexes: effects on embryo development and nuclear lamin

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Keywords: cattle, cryopreservation, cryoprotectant.

Compromised developmental competence after oocyte cryopreservation is due to damage in important structures. Nuclear lamins provides mechanical stability, nuclear shape and is involved in several processes such as DNA replication and repair. The aim of present study was to examine the effects of cryoprotectant exposure (experiment I) and vitrification by OPS (experiment II), of immature bovine Cumulus-Oocyte Complexes (COCs) on embryo development and structure of nuclear lamin. Ovaries were obtained from the local slaughterhouse. Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for experiments. Base medium for preparation of cryoprotectant solutions was holding medium (HM), which consisted of HEPES-buffered TCM199 supplemented with 20% newborn calf serum. COCs were first exposed to HM for 3 min followed by first vitrification solution: 7.5% dimethylsulfoxide (DMSO) and 7.5% ethylene glycol (EG) in HM for 15 min. Then, oocytes were transferred to two drops of second vitrification solution: 16% DMSO, 16% EG and 1 M sucrose in HM for 20 s each. For warming, the oocytes were submerged for 1 min in warming solution 1 (HM plus 1 M sucrose) and transferred to warming solution 2 (HM containing 0.5 M sucrose) for 3 min and washed twice in HM for 5 min. Thereafter, COCs were processed for *in vitro* maturation, fertilization and culture. Samples of COCs were fixed and immunostained for oocyte nuclear lamin evaluation. For statistical analyze, Chi-square test was used to compare data among the experimental groups ($p<0.05$). Exposure of immature COCs exhibited lower blastocyst rates 29% (31/108) compared to the control 51% (101/199). Higher hatching rates were observed in control 22% (43/199) as compared to vitrified 6% (7/108). Organization of nuclear lamin in immature COCs exposed to cryoprotectants exhibited a higher proportion of contractions and morphological alterations (35%) compared to control group (0%, $p < 0.05$). The vitrification by OPS affected developmental competence showed higher of cleavage in control group 79% (203/256) than in vitrified group 8% (13/156). COCs of control group showed blastocyst development of 42% (107/256) whereas no blastocyst was observed in vitrified group. Nuclear lamins was strongly affected by vitrification in immature oocytes, exhibited higher proportion of alterations (72%) compared to control group (9%; $p < 0.05$). In conclusion, immature COCs seem to be better tolerant to the cryoprotectant exposure, however, vitrification result in low embryo development due, probably to negative effects on nuclear lamin.

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A314 Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Sperm storage tubules culture: a new approach for reproductive research in avian species

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Keywords: cell culture, avian, epithelial cells, sperm storage tubules.

Sperm storage tubules (SST) are epithelial structures found in the mucosa of distal half of the oviduct of all avian species studied. These tubules maintain and store sperm up to 70 days and this allows fertilization without insemination. The aim of this work was to set up epithelial SST cells culture for future use as an *in vitro* model for oviduct cells-sperm interaction. Hens (*Gallus gallus domesticus,* Unité de Recherches Avicoles [URA], INRA, Nouzilly.) were euthanized with sodium pentobarbital injection. Oviducts were isolated and removed and the uterovaginal villi was manually dissected under stereomicroscopy. The SST area on the top of isolated mucosal villi was dissected, scalped in small fragments, and enzymatically digested in 1µg/ml Collagenase for 10 min at 41ºC. The digested tissue was flushed for 30 times by pipetting. The enzymatic activity was blocked by washing the tissue twice with culture medium. A second enzymatic digestion was performed by incubating the tissue overnight at 4ºC in 1µg/ml Pronase. The tissue was flushed again and the enzymatic activity was blocked. SST were isolated in 2 / 4% Percoll density gradient centrifugation at 2000g for 30 min at 4ºC. An intermediate phase of Percoll column containing SST was harvested before being maintained in Medium 199 containing 10% BFS and Gentamicin, during 30 min at 41ºC for fibroblast attachment. The medium containing SST was distributed in Lab-Tek Chamber Slide System (Nunc). SST were cultured at 37ºC, 5% CO_2 atmosphere, for 6 days. Immunocytochemistry for epithelial cell type confirmation, was performed with overnight incubation with monoclonal primary antibodies anti-Pan-cytokeratin (1:300, Sigma), Tubulin (1:300, Sigma) and Vimentin (1:500, Sigma) and anti-species secondary antibodies. We observed that, at the end of the enzymatic process, 90% dissected SST was isolated. In phase contrast microscopy we observed integral SST as well as individual cells. After 2 days of culture we observed cell migration from SST borders to form a monolayer. Eighty % cells presented epithelial characteristics as demonstrated with Cytokeratin and Tubulin positivity and Vimentin negativity, in Confocal microscopy. The digestion and isolation processes need to be controlled to differentiate the epithelial surface mucosal cells from SST cells. This method is very effective to isolate the SST specific population of cells that can be used in different reproductive and physiological studies for epithelial cell-sperm interaction.

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A315E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Live confocal microscopy time-lapse imaging cholesterol inclusion to plasma membrane of mature bovine oocytes prior to vitrification

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Keywords: bovine oocytes, cholesterol, plasma membrane, confocal microscopy time-lapse.

The present study aimed to evaluate the effect of 2 mg/mL of methyl-β-cyclodextrin (MβCD) as a cholesterol loader to change mature oocyte plasma membrane and increase its tolerance to vitrification procedure. In a first set of experiments, a time-lapse imaging by confocal microscopy was conducted to determine at which time the MβCD loaded the cholesterol in the plasma membrane. Once the timing of cholesterol integration to plasma membrane was defined, we evaluated the effects of a pre-treatment with 2 mg/mL of MβCD for 35 min and 1h prior to vitrification on survival and embryo development rates. Analysis in all groups were performed through an ANOVA, followed by the Sidak's post-hoc test. In all cases, significant level was set at $P < 0.05$. *In vitro* matured oocytes exposed to 2 mg/mL of MβCD showed a clear immunofluorescence in the plasma membrane after a minimum of a 35 min and up to 1h pre-treatment. Higher survival rates were observed when oocytes exposed to 2 mg/mL of MβCD pre-treatment for 35 min (60.6%) were vitrified/warmed compared to 1h pre-treatment (47%). Similar results were observed when blastocyst yield was determined. Blastocyst rate on D7 was higher after 35 min MβCD treatment compared to the 1h MβCD treatment (3.1% vs 0%). However, vitrified oocytes showed lower embryo development rates than fresh non-vitrified oocytes (21.1%). Hence, our results warrant further research to be conclusive.

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A316E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Recipient pregnancy rates after transfer of vitrified *in vivo* produced ovine expanded or hatched blastocysts

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Keywords: blastocysts, pregnancy, sheep, vitrification.

The development of embryo transfer technology pushed freezing technology in domestic species. Controlled (traditional) slow freezing and vitrification (ultra-rapid freezing) have been the two major techniques used for embryo cryopreservation (Fahy and Rall, 2007). The ultra-rapid technique, such as vitrification, has reduced time and cost of the procedure since it does not require any special equipment and is, therefore, well adapted to routine field use (Baril, 2001). Sheep and goat embryos are able to survive vitrification procedures and with further research this method may provide an economical alternative to the current freezing methods. Shirazi et al., (2010) indicated that the survival of IVP ovine embryos following vitrification progressively increases as the developmental stage of the embryo proceeds. We studied the effect of vitrification on the developmental stage of the embryo monitored after ET. Donor ewes (10 Romanov breed) were used to produce vitrified embryos kept under semi-extensive husbandry conditions and fed on a maintenance diet (ARC 1990). Superovulation was induced by treatment with ovine follicle stimulating hormone, FSH (Ovagen, Immuno-Chemical Products Ltd) that was administered in 8 equal doses at 12-hourly intervals (total dose equivalent to 9 mg NIADDKoFSH- 17) commencing 60 hours prior to the end of progestagen treatment (12 d). Embryos were recovered by the surgical procedure on day 6 following insemination and were assigned on their developmental stage and quality grade according to standards of the International Embryo Transfer Society (Savoy, IL). Within 2 to 4 h after collection, expanded (n=18) and hatched (n=18) blastocysts were first washed in phosphate-buffered saline (PBS) supplemented with 0.5 mM sodium pyruvate, 3.3 mM glucose and 10% FCS. The embryos were vitrified at room temperature (~23 °C) as follows: 10% EG+10% DMSO for 3 min, followed by 20% EG+20% DMSO+0.5 M Sucrose for 30 s, loaded into OPS and directly plunged into LN2. Before ET the embryos were warmed directly by plunging them into tissue culture medium-199 (TCM-199) + 20% foetal calf serum (FCS) at 37 °C for direct dilution. Following the direct dilution, the embryos were transferred as single into synchronised recipient ewes and allowed to go to term. Pregnancy rates were assessed by ultrasound scanning at 50 days after transfer. We used the *chi-square* test to compare *pregnancy rates*. Late stage embryos produced in vivo to the expanded blastocyst stage before cryopreservation had a significant ($P<0.05$) higher (86%) of pregnancy rate than those recorded (60%) for the hatched stage blastocyst. Our results were higher than the results reported by Garcia-Garcia et al., (2005). They worked with in vivo produced ovine embryos in early stages (2-12 cells) and cultured to the blastocyst stage and frozen thereafter. In that study it was reported that blastocyst stage of embryos had a significantly higher viability than their counterparts frozen at earlier cleavage stages, (66.1% versus 23.1%). The results indicate that in vivo produced embryos up to expanded blastocyst stage can be successfully cryopreserved by vitrification while vitrification success decreases when the stage of development reaches hatched blastocyst.

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A317E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Effect of the rewarming temperature on survival rate of IVP bovine embryos vitrified in triacetate cellulose hollow fiber incorporated into a new vitrification device

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Keywords: IVP bovine embryos, rewarming temperature, triacetate cellulose hollow fiber, vitrification.

Hollow fiber vitrification (HFV) was introduced by Matsunari et al. (J. Reprod. Dev. 58: 599–608, 2012) as a method for vitrifying groups of mammal embryos and was shown to be effective for cryopreservation of such cryosensitive objects as in vivo and in vitro produced porcine morulae. Due to the standard and simple vitrification procedure HFV method may be perspective for cryopreservation of bovine oocytes and IVP embryos in combination with embryo transfer methods. The objective of this work was to introduce a vitrification device that will allow effective storage of the triacetate cellulose hollow fibers (HF) that become fragile in liquid nitrogen and to show effectiveness of the HFV method for IVP bovine embryos rewarmed at room temperature (22–24°C). IVP bovine embryos were cultured in modified SOF medium. Morphologically normal blastocysts and expanded blastocysts were collected at day 7 after IVF and used for vitrification. A vitrification device was constructed by connecting a piece of HF to a heat-pulled tip of a glass capillary. A protective sheath was fitted directly on the capillary. Embryos in groups of 5–10 were loaded into the vitrification devices in the equilibration solution containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO). Then HFs were exposed to vitrification solution containing 15% EG, 15% DMSO and 0.5M sucrose for 1 minute and immersed into liquid nitrogen. HFs were rewarmed in the solution with 1M sucrose at either 39 °C or 22–24°C and were transferred into dilution and washing solutions stepwise. Rewarmed blastocysts were cultured for 72 hours in modified SOF medium. Reexpansion and hatching rates of embryos were assessed at 24 and 72 hours post rewarming, respectively. Results are presented as mean ± SD. Data was analyzed using Student's t-test. Significance was set at $p < 0.05$. After storage in liquid nitrogen for 2–12 months and transportation to the farm all HFs within vitrification devices remained intact. Volume of vitrification solution with loaded blastocysts within hollow fibers ranged between 0.024 and 0.030 mkl. Reexpansion rates of the blastocysts after rewarming at 22–24°C and 39°C were 82.65 ± 11.62% (159/191; 14 repeats) and 88.73 ± 5.99% (64/73; 7 repeats), respectively. Hatching rates were 53.77 ± 22.37% after rewarming at 22–24 °C and 64.74 ± 12.74 % at 39°C. There were no statistically significant differences between two experimental groups. The introduced vitrification device is relatively simple in construction and protects HFs with loaded embryos from mechanical damage. Due to the very small volume of the samples within HFs, rewarming at room temperature did not significantly affect survival rate of the embryos. Rewarming at 22–24°C can be advantageous for practical uses and may help to avoid temperature related effects of high concentrations of cryoprotectants, such as DMSO.

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A318E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Born Simmental calves after the transfer of genetic evaluated day 7 bovine embryos

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Keywords: cattle, day 7 embryos, genetic evaluation, Simmental calves.

Genomic selection can theoretically take place in any stage of an animal's life. The use of ET can increase the intensity of selection, but at the cost of keeping many recipients. This can be avoided by transferring only embryos with desired gender and best breeding values. Improvement of embryo micromanipulation and DNA amplification techniques allows for the direct genetic analysis of bovine embryos prior to implantation. The aim of our study was to set up and optimize a whole embryo production and evaluation line in Simmental cattle to determine gender, polled status, hereditary defects and reliable breeding values on blastomeres at the morula and blastocyst stages. For embryo recovery (n=45) German Simmental animals (n=17) were superovulated using a standard protocol. Embryos were biopsied immediately after recovery by a single operator under a mobile stereo microscope (Olympus) at 50x magnification with a single use special steel blade mounted on a blade holder (Bausch & Lomb, Germany) attached to a micromanipulator (Eppendorf, Germany). Two biopsy methods were compared, first embryos were splitted and one third of a half cut off (G1, n=161) or by cutting of the trophoblast (G2, n=146). Biopsied cells, approximately 10-15, were immediately used for whole genome amplification (*Repli-g* mini Kit, Qiagen) followed by PCR analysis of gender and polledness. Hereditary defects were analyzed using a 5'-exonuclease assay. Embryos were transferred to recipients after *in vitro* culture in SOF supplemented with 5% ECS, 40 µl/ml BME and 10 µl/ml MEM in four-well dishes, under mineral oil, at 39°C and gas mixture (5% CO2, 5% O2, 90% N2) for 24 h. DNA of the first 14 born calves was extracted from blood samples. These calves together with the corresponding embryos were genotyped with the Illumina Bovine 54k BeadChip. Call rates were recorded, correlations between embryo and calf genotypes calculated and breeding values estimated. The biopsy technique G1 resulted in the highest number of good quality transferable embryos G1 (1.37) vs. G2 (0.97) ($p<0.05$) in relation to the number of original embryos. However, better pregnancy rates were obtained by transferring 2 demi-embryos to one recipient (1 demi-embryo=28.6%; 2 demi-embryos=76.2%). Biopsy technique G2 resulted in 55.0% pregnancies. No discrepancies could be detected between gender, polled and hereditary defect status of born calves and corresponding embryos. The average call rate for the genotyped embryos was 0.922, ranging from 0.841 to 0.980. The call rate of the corresponding calves ranged from 0.998 to 0.999. The average concurrency of the obtained genotypes of embryos and calves was 98.7%, with an average correlation of 0.991. Gender, polledness and genotypes obtained from preimplanted embryos were consistent with genotypes obtained of the born calves. Therefore, our first results provide promising prospects for the optimized production line.

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A319E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Comprehensive proteomic analysis of *Gallus gallus* uterine fluid

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Keywords: *Gallus gallus,* proteomic analysis, uterine fluid.

Domestic hens are able to keep spermatozoa in their genital tract for long periods, and can so produce fertilized eggs for up to 3 weeks after one insemination. An extensive description of the avian uterine fluid proteome will help to provide the basis for a better understanding of a number of diseases and processes, including sperm survival but also female infertility and cell storage. Uterine fluid was collected (n=10) into a plastic tube placed at the entrance of the everted vagina 10h after oviposition. Bottom up proteomic approach using SDS-PAGE and nano LC-MS/MS (ultimate 3000 RSLC system coupled to LTQ Velos Orbitrap mass spectrometer) was performed with a high-low resolution MS strategy. Data were matched against NCBInr database using Mascot 2.3 and identifications were validated by the peptide and protein Prophet algorithm using Scaffold 4.0 software. Bioinformatics treatments of data set was carried out to refine annotation of proteins using NCBInr database, and to describe uterine fluid proteins using SecretomeP 2.0 and SignalP 4.1 tools, InterproScan software, and, Exocarta, KEGG and UniprotKB databases. Among a total of 922 proteins that were identified, 836 (91%) were identified in *Gallus gallus* databases, whereas 86 (9%) were identified in others species, indicating unknown chicken isoforms. Deepens analysis of cellular component revealed three categories of proteins. The secreted proteins (165) known to be secreted with a peptide signal or by an unconventional pathway, the exosomal proteins (644) which match against exosomal databases (Exocarta, UniprotKB, KEGG) and the last category refers to proteins which are not annotated as exosomal or secreted (113). Secreted proteins are composed of protease inhibitors (11), cytoskeletal and extracellular matrix proteins (22), enzymes (metabolic, proteases etc.) (49) and others proteins implied in calcification of eggshell (OC-17, OC-116). Exosomal proteins mainly consist in enzymes (metabolic, oxidoreductase) (225), chaperon proteins (HSPA8, HSP90AA1,...) (26) and proteins implied in MVB biogenesis (Alix, TSG101, Clathrin,…) (25). We have isolated exosomes and confirmed the presence of exosomal markers (CD63, HSPA8) by western blot in the avian uterine fluid. The presence of exosomal proteins in avian uterine fluid may represent a novel and exiting mechanism of cell-cell interactions, that may explain at least in part, the long term sperm survival. We believe that the thorough catalogue of proteins presented here can serve as a valuable reference for the study of sperm interaction with the female genital tract. Moreover, it could be an interesting tool for biomarkers discovery involved in fertility.

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A320E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Assessment of fertilizing ability of Iberian ibex (*Capra pyrenaica*) vitrified and frozen epididymal sperm by *in vitro* heterologous fertilization of bovine oocytes

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Keywords: bovine oocytes, epididymal sperm, goat, heterologous fertilization.

The aim of this study was to evaluate the fertilizing ability of vitrified and frozen Iberian ibex sperm by assessing heterologous IVF using bovine oocytes. Testes were obtained from mature ibexes that were legally hunted in the Tejeda and Almijara Game Reserve, in southern Spain. Epididymal spermatozoa were collected by the retrograde flushing method. Sperm from right epididymis was vitrified with TCG-6% egg yolk plus 100 mM sucrose while sperm from left epididymis was conventionally frozen with TCG-6% egg yolk and 5% glycerol. *In vitro* matured zona-intact bovine oocytes were subjected to heterologous IVF with vitrified-warmed (n=495) or frozen-thawed ibex sperm (n= 565) and homologous IVF (n=299). A non-fertilized group was included as control for parthenogenesis (n=81). For heterologous fertilization, sperm pool of three males was used for each treatment. Sperm-oocyte interactions were evaluated at 2.5 hours post-insemination (hpi) by the number of attached and bound spermatozoa whereas penetration and polyspermy were evaluated after 12 hpi. Presumptive zygotes were fixed and stained with Hoechst 33342 at 18, 20, 22, 24 and 26 hpi to assess pronuclear formation using a phase contrast and confocal microscopy. Besides, cleavage rate was evaluated in all groups at 24 hpi. Data obtained was analyzed using one way ANOVA (Sigma Stat, Jandel Scientific, San Rafael, CA) Results showed a higher number of bound and attached spermatozoa in both heterologous groups compared to homologous group ($P<0.001$). The homologous IVF group as expected, showed the highest percentage of pronuclear formation at 18 hpi (67.7±9.8%), significantly different to both heterologous groups (Frozen: 21.3±13.9%; Vitrified: 28.8±15.5%, $P<0.05$). Indeed, pronuclear formation was delayed in both heterologous groups with the highest percentage at 24 hpi (30.3 ± 15.1%) for frozen sperm and at 20 hpi (31.7 ± 21.5%) for vitrified sperm. In addition, cleavage rate was higher in homologous group compared with heterologous frozen and vitrified groups (76.1±15.9% *vs*. 31.3±27.2% and 45.1±24.4%, respectively, $P<0.05$). No differences were observed between heterologous vitrified and frozen sperm in all parameters evaluated. In conclusion, Iberian ibex epididymal sperm can be vitrified successfully, maintaining its fertilization ability in the same extend as frozen sperm. To our knowledge, this is the first report of successful epididymal sperm vitrification in a mammal species being capable of fertilization as a standard tool for genome conservation in threatened species.

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A321E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Cryopreservation of sheep embryos by slow freezing or vitrification with or without caffeic acid

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Keywords: antioxidant, caffeic acid, cryopreservation, plasminogen activator activity, sheep embryos, slow freezing, vitrification.

Reduced viability of embryos after cryopreservation has been associated with lipid peroxidation due to increased levels of free radicals. Thus the addition of antioxidants in the cryoprotectant solutions might be beneficial to embryo survival. Antioxidant caffeic acid, that has been shown to reduce the levels of lipid peroxidation markers in rat erythrocytes, was tested for its ability to improve the cryotolerence of sheep embryos under the two major methods used for embryo cryopreservation. Embryos were collected from 32 superovulated Lesvos ewes, on day 7 after the onset of oestrus, soon after slaughter, by uterine horns flushing and were evaluated under stereoscope. One hundred and thirteen freezable embryos (grades 1, 2), in the morula or in the blastocyst stage, were cryopreserved either by slow freezing (seeding at -6.5°C, 0.3°C/min to -35°C) or by vitrification. Unless differently specified, all chemicals were purchased from Sigma-Aldrich Co. (St Luis, MO, USA). Ethylene glycol, in a final concentration of 1.5M in ECM [Embryo Culture Medium = PBS+20%FCS (Biochrom AG, Berlin, Germany)], was used as cryoprotectant in slow freezing. A final concentration of 25% glycerol and 25% ethylene glycol in ECM was used for vitrification. In half of the cases in each method, 20μM of antioxidant caffeic acid was added in all the cryoprotectant solutions. After thawing / warming, the embryos were cultured in vitro, in SOF, for 72 hours and evaluated for development and hatching. Plasminogen activator activity (PAA), which has been linked to embryo development or degeneration, was determined spectrophotometrically in the media used during the removal of cryoprotectants and in vitro culture. Data was analysed using chi square test, t-test and regression analysis. Overall, 56.0% of the thawed / warmed embryos developed during in vitro culture. At the end of in vitro culture, 42.0% of all the incubated embryos were undergoing or had completed hatching; 42.3% after slow freezing and 41.7% after vitrification. Increased hatching ratio was observed in the embryos cryopreserved in the presence of caffeic acid (52.0% vs. 32.0%, $P<0.05$); this was apparent in both cryopreservation methods and the difference approached significance after slow freezing (53.8% vs. 30.8%, $P<0.10$) but not after vitrification (50.0% vs. 33.3%, $P<0.20$). At the end of in vitro culture, 47.0% of the embryos were degenerating; no statistically significant effect of cryopreservation method or the presence/absence of caffeic acid was observed. PAA in the culture medium at the end of in vitro culture was negatively associated with the ratio of degenerated embryos ($R2=0.465$, $P<0.05$). In conclusion, addition of antioxidant caffeic acid seems to improve cryotolerence of sheep embryos and its effect seems to be more prominent when slow freezing is applied.

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A322E Cryopreservation and cryobiology, diagnosis through imaging, molecular biology and "omics"

Vitrification of intact and splitted *in vitro* produced d7 bovine embryos

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Keywords: cattle, embryo splitting, embryos, IVP, vitrification.

In the present study, we compared the vitrification of intact and splitted *in vitro* produced bovine embryos with two vitrification methods: The CryoLogic Vitrification Method (CVM), CryoLogic® Australia and the Hollow Fiber Vitrification (HFV) Method (Matsunari et al. 2012). For IVP ovaries from slaughtered animals were used. Aspirated oocytes were *in vitro* matured (IVM) for 22 h, followed by *in vitro* fertilization (IVF) for 18 h. Presumptive zygotes were denuded and *in vitro* cultured (IVC) in SOF supplemented with 5% OCS. On D7, intact or splitted embryos were classified in grade I or II and vitrified, either by the CVM or by the HFV method. Embryos were loaded in 0.7-1.0 µl of vitrification solution. Vitrification and thawing procedures were performed as previously described (Saucedo et al., 30th Annual Meeting A.E.T.E., Dresden, Germany, 2014). After thawing, embryos were *in vitro* cultured until D12. Survival rate (judged by re-expansion) 24-48 h after thawing and hatching rate were recorded. Within the HFV method 273 (intact: HFV-) and 50 (splitted: HFV+), and within the CVM method 256 (intact: CVM-) and 312 (splitted: CVM+) embryos were cryopreserved. The percentage of lost embryos was lower in HFV- (0.7%) vs. HFV+ (9.0%) vs. CVM- (9.2%) vs. CVM+ (16.4%). The overall re-expansion rate was significantly higher with CVM than HFV (70.8 vs. 61.0%; $p>0.05$) and the highest results were obtained with blastocysts (73.6%) followed by early blastocysts (70.8%) and morulae (58.0%) ($p=0.004$, Kruskall-Wallis test). No significant differences were observed using intact or splitted embryos. Re-expansion rate of intact embryos resulted in 68.6% vs. 68.8% of splitted embryos ($p=0.835$; Mann-Whitney test). Survival of embryo regarding the time of culture between splitting and vitrification (3 or 20 h) showed a tendency to highest results after 20 h (63.0% vs. 72.1%; $p=0.807$, Mann-Whitney test). Demi-embryo survival and effect of embryo's stage on biopsy outcomes were evaluated. No significant difference was found among stages (59.0%, 67.2%, and 90.0%, for morulae, early blastocysts and blastocysts, respectively; $p=0.0568$ Kruskal-Wallis test) with regards to survival after splitting and biopsy. However, blastocysts leads to better survival after splitting and vitrification. In conclusion, both vitrification methods are suitable for intact or splitted bovine embryos, whereas the CVM seems to be more practical in handling.

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